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ANNUAL REPORT

DIVISION OF CANCER ETIOLOGY

NATIONAL CANCER INSTITUTE

October 1, 1984 through September 30, 1985

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ANNUAL REPORT OF

THE LABORATORY OF BIOLOGY

CHEMICAL AND PHYSICAL CARCINOGENESIS PROGRAM

NATIONAL CANCER INSTITUTE

OCTOBER 1, 1984 through SEPTEMBER 30, 1985

The goal of the Laboratory of Biology is to elucidate cellular alterations occurring during carcinogenesis in order to identify the series of steps that lead to malignancy. The primary objective is to determine the crucial molecular and physiological changes that occur in cells, which have been treated with chemical or physical agents, as they transform from the normal to the neoplastic state. Coordinated biochemical and biological studies are used (1) to characterize cellular alterations associated with carcinogenesis, (2) to evaluate relationships between DNA metabolism and carcinogenesis, (3) to determine effects of physiological host factors on carcinogenesis, and (4) to develop new in vitro cellular transformation systems which are pertinent for the study of the molecular mechanisms of carcinogenesis.

The major emphasis of the Laboratory of Biology concerns the identification of relevant alterations in target cells that result in malignancy. For this purpose several different mammalian cell model systems have been used; the models, for the most part, have originated in the laboratory. Because of our ability to obtain neoplastic conversion of diploid cells with our transformation models, we are directing our efforts toward understanding the basic molecular events occurring during transition to malignancy. The application of gene cloning and flow cytometry has added new dimensions to the study of stages of carcinogenesis. For example, we are currently analyzing guinea pig and hamster chemically transformed lines for activation of cellular proto-oncogenes. In addition, human cells exposed to known carcinogen insult are being cataloged in terms of cell surface changes shared with spontaneous human malignant cells.

The major objective of the Somatic Cell Genetics Section is concerned with the relevant changes in chromosomes and DNA metabolism which regulate gene expression responsible for neoplastic transformation. By superimposing molecular events on biological observations, it should be possible to make conclusions concerning gene expression relevant to control of differentiation and cancer. The Tumor Biology Section emphasizes host interactions, particularly from an immunological point of view. The objective of these studies is to examine phenotypic changes at the cell surface to identify the effectors biochemically and to study their mode of action.

A few in vitro mammalian cell models are available for investigating cellular and molecular mechanisms of chemical carcinogenesis and for determining the potential carcinogenicity of environmental chemicals. Although the results obtained with these models correlate well with results from lifetime studies with experimental animals and human epidemiologic data, similar end points have not been obtained with human cells from normal sources or from individuals with inborn errors of metabolism associated with higher risks of malignancy. The results with human cells suggest that a difference in control mechanisms at the

target cell level is responsible for "competence" that makes the cell susceptible to transformation by a carcinogen. Contrary to rodent cells which are transformed by a variety of carcinogens, human cells subjected to the same carcinogens exhibit neither indefinite proliferation nor loss of growth control. Furthermore, the chromosomal defects found in human cells after carcinogen treatment are either not as extensive or lack the specific defect which is associated with indefinite proliferation or loss of growth control usually associated with mammalian cell transformation.

The information gained from the study of rodent cells in vitro is of limited value in application to attempts to transform human cells. Cell survival and metabolic studies indicate that carcinogens are metabolized by human cells in vitro. Chemical carcinogen concentrations effective in inducing transformation in animal cells also increase the frequency of SCE and chromosome aberrations in human cells. Yet, only rarely are normal human cells converted to the malignant state after carcinogen exposure. These results are similar to those from experiments that utilized cells of other mammalian species such as dog, opossum, or monkey. Human cells possess control mechanisms that are responsible for a stable phenotype that cannot be altered readily by one or a series of a few carcinogenic insults. For example, some data indicate that human cells have a capacity to efficiently carry out unscheduled DNA synthesis after exposure to ultraviolet light; other types of cells, such as hamster, can survive with unrepaired damage. Furthermore, the chromosomal defects found in human cells after carcinogen treatment are either less extensive or lack the specific defects associated with continued cell proliferation or loss of growth control commonly associated with malignant cells.

The different cancer sites may have tumors originating from either epithelial or fibroblast cell types. Embryonic and mesenchymal tumors with specific types of leukemias and forms of sarcomas constitute the largest percentage of cancers during the first two decades of life. Subsequently, the frequency of carcinomas increases greatly, and respiratory and digestive system carcinomas increase significantly. Probably the simplest explanation for this biphasic response is the alteration in cell cycle which occurs with aging. Although fibroblast cells rarely cycle in adults, continuous but regulated stem cell cycling of epithelial type cells occurs at a rate allowing replacement of epithelial surfaces. Therefore, the use of fibroblasts, as well as epithelial cells, in vitro are relevant to the study of carcinogenesis.

Human papilloma virus-16 DNA has been transfected into NIH 3T3 cells. This achievement provides a useful model for studying the transforming functions of HPV 16 DNA. Data presented clearly establishes that the HPV 16 DNA is present within the cells, in multiple copies, and as with human cervical cancer is primarily integrated into the host DNA. The importance of this virus integration is its association with cervical carcinoma. For this reason the role of early gene expression in establishment of the transformed state is under analysis. The E6 gene which is considered to be a transforming gene for bovine papilloma is of particular interest. Enzyme cleavage within HPV 16 DNA at a site close to the C-terminal end of E6 gene indicates a reduced in vivo tumorgenicity of the transformed 3T3 cells. Poly A+ RNA analysis also indicates that transformation is accompanied by expression primarily of early genes. The information obtained utilizing the NIH 3T3 model for transformation is being used in a new approach for obtaining human cell malignant transformation. There is both experimental and clinical evidence to indicate that papilloma viruses

can act as cocarcinogens with both physical and chemical carcinogens. Therefore, the plasmid containing the HPV 16 DNA has been transfected into a non-tumorigenic human fibroblast line that was originally transfected using an origin defective SV40 construct and into fibroblast and epithelial cells obtained from human foreskin. Southern blot analysis of the extracted DNA indicates that HPV sequences can persist in both fibroblast and epithelial cells.

The treatment of embryonic lung-derived fibroblasts, MRC-5 cells, by carcinogen alone, either physical or chemical, has not resulted in any permanent cell lines. Cytogenetic analysis of these cells indicated relatively few chromosome alterations and consisted primarily of structural changes. No specific alterations were obtained; however, these cells proliferated approximately twice the number of doublings of non-treated cells. The possibility is considered that the chromosome changes that have been observed although not relevant for malignancy were responsible for the extended proliferation phase of the cells. Cytogenetic analysis by G-banding of 10 cell lines revealed that 7 were aneuploid with both structural and numerical chromosome alterations; the other 3 were normal diploid. Structural alterations consisted of chromosome deletions, translocations, and partial chromosome duplications. Although no specific structural or numerical abnormality has been detected, several structural alterations involved chromosome 1 at the _src_ and _fgr_ loci, and chromosomes 7, 11, and 22 at _erb-B_, H-_ras_-1 and _sis_ loci, respectively. A homology exists between some of these proto-oncogene sequences and genes involved in cell growth regulation. These chromosome changes may be responsible for the extension of the life span of the treated cells (2-3 fold) relative to untreated cells. Several sarcomas obtained from surgical biopsy were also chromosomally analyzed. Compared to _in vitro_ transformed cells, these tumors, which are derived from the same cell type, have considerably more extensive chromosome alterations. Although all the carcinogens used caused chromosome damage, a relatively low frequency of chromosome alterations persisted at advanced passages after carcinogen exposure. Carcinogen-induced cell alterations may represent a step in the process of neoplastic development. The extent of the structural alterations observed in _in vitro_ exposed cells compared to those detected in sarcomas or virally transformed cell lines suggests that additional genetic alterations including chromosome changes are necessary for a complete expression of malignancy and indefinite proliferation.

The Syrian hamster fetal cell model continues to be pivotal to the study of the underlying mechanisms that control the modulation of _in vitro_ transformation by a variety of carcinogens. The dose response relationships and the ability to demonstrate tumorigenicity indicate the relevance of this model to cancer. The role of O^6-methylguanine in the initiation of carcinogenesis by methylating agents has been assessed. The persistence of O^6-alkylguanosyl residues in tissues susceptible to carcinogenesis by alkylating agents has led to the hypothesis that they are the critical lesion for the initiation of carcinogenesis. With the hamster model, it was possible to quantitatively compare the induction of transformation, cell lethality, and DNA lesions with three different methylating agents: N-methyl-N'-nitro-N-nitrosoguanidine, N-methyl-Nnitrosourea, or methyl methanesulfonate. Very different molar quantities of each agent were required to induce equivalent levels of transformation. With these concentrations, the level of O^6-methylguanine but not N^7-methylguanine induced was similar. Both O^6- and N^7-methylguanines were removed from the DNA by hamster cells to a limited extent. The frequency of transformation relative to O^6-methylguanine induction is much greater than that of known mutation

frequencies. The target size for carcinogenesis was calculated at a minimum of 10^4 nucleotides. This suggests that either one of many genes can initiate carcinogenesis or that the initiation is not the result of a single base mutation. Hamster cells can also be transformed by etiological agents for which there is no indication of DNA interaction. Bisulfite (SO_2) is an example currently under study. At neutral pH this compound is mutagenic to neither mammalian nor bacteria cells but does induce dose dependent, morphological transformation of hamster cells. No detectable effect on excision of post-replication repair was noted. Transformed cells were isolated and tumor producing lines have been obtained. Coordinated, quantitative and qualitative polypeptide differences were demonstrated among all neoplastic lines that had been initiated by sodium bisulfite. Only malignant lines had break protein distributions reflecting the quantitative changes. The qualitative changes were considered to be either early transformation events or those more closely associated with the acquisition of tumorigenicity. In the final analysis, the qualitative polypeptide changes found in the bisulfite-induced malignant lines were similar to those seen in a benzo-[a]-pyrene induced malignant line. The latter suggests that there is a convergence of pathways responsible for carcinogenesis independent of the nature of the initiating agent.

Activation of proto-oncogenes is being studied in two model systems: the hamster and the guinea pig. Activated oncogenes have been identified by foci formation after transfection of DNA into NIH 3T3 cells from a number of hamster transformed lines. A number of transformed foci contain hamster DNA as evidenced by presence of hamster intracisternal A particle sequences (IAP). Further analysis of these lines indicates that the hamster activated oncogenes are not related to H-ras, K-ras, bas, myc, fos, mos, src, or abl. In the case of one transformed line, the activated oncogene is closely linked to a hamster IAP sequence. An activated oncogene has been molecularly cloned from a guinea pig transformed line. This same oncogene is activated in four other independently isolated lines. Activation is closely associated with tumorigenic potential and was independent of the initiating carcinogen. The biologically active cloned oncogene has weak homology with H-ras. However, further analysis of the DNA and RNA expression indicates that this is an oncogene heretofore that has not been described previously.

Lymphokine preparations prepared from antigen or mitogen stimulated lymphocytes contain a potent anticarcinogenic activity which is the property of a new lymphokine termed leukoregulin. Furthermore, leukoregulin induces specific target cell plasma membrane changes accompanying its natural killer cell sensitizing and tumor cell proliferation inhibitory activities. The membrane changes can be rapidly detected by flow cytometric analysis of both light scatter and membrane permeability changes, the latter being followed by the uptake or by the loss of intracellular fluorescent molecules such as fluorescein or propidium iodide, respectively. The same changes are observed in target cells during the course of natural killer cell cytotoxicity. This suggests that leukoregulin may be an intrinsic mediator or element of natural lymphoid cell cytotoxicity and occupies a central role in immunological homeostasis.

Cells in all phases of the cell cycle are sensitive to the proliferation inhibitory action of leukoregulin. Flow cytometric analysis of human K562 erythroleukemia cells treated with increasing concentrations of leukoregulin to inhibit replication of more than ninety percent of the cells demonstrates that the percentage of cells in each phase of the cell cycle remains constant during

seventy-two hours of leukoregulin treatment. Fluorescence activated cell sorting of leukoregulin plasma membrane permeability altered cells also reveals that cells in each phase of the cell cycle are susceptible to the membrane perturbing action of leukoregulin. Flow cytometric analysis of the sorted cells, moreover, indicates that cells in the "S" phase of the cell cycle may be more sensitive to the acute membrane destabilizing action of leukoregulin. This cell cycle independent pattern of inhibition of cell replication and its rapid reversibility are quite different from the cell cycle phase specific blocks observed with the typical inhibitors of cell replication affecting protein and/or nucleic acid synthesis.

The molecular events underlying the perturbation in plasma membrane stability and permeability following target cell interaction with leukoregulin have been studied by contrasting the changes in leukoregulin treated cells with those after exposure of target cells to a variety of membrane active agents affecting ion transport. Compounds including oubain, amphoteracin B, calcium ionophores A23187 and X-537A, phospholipasae C and A2, and phytohemagglutinin which increase intracellular calcium levels mimic the membrane changes induced by leukoregulin. Calmodulin, calcium channel blockers and both sodium and potassium ionophores and channel blockers exhibit no flow cytometrically detectable membrane destabilizing activity. The calcium stimulators are active over a wide range from 10^{-3} to 10^{-10}M and the one paralleling the kinetic activity of leukoregulin most closely is the calcium ionophore A23187. This suggests that leukoregulin may exert its anti-cancer action in part by altering intracellular calcium levels. Furthermore, measurement of intracellular ionic calcium using the calcium binding fluorescent probe quin2 reveals a transient increase in intracellular calcium approximately 10 minutes after treatment of human K562 erythroleukemia cells with leukoregulin. Treatment of the cells with calcium ionophore A23187, however, produces an increase in intracellular calcium within one minute of ionophore exposure indicating that although leukoregulin may alter calcium levels it does not function solely as an ionophore. Newer and more specific fluorescent calcium indicators are being used to further define the bioactivity of leukoregulin and other lymphokines in terms of calcium metabolism and membrane stability and their relationship to preventing carcinogenesis and inhibiting the proliferation of neoplastically transformed cells.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP04629-20 LB

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Regulation of Stages of Carcinogenesis Induced by Chemical or Physical Agents

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P.I.:	J.A. DiPaolo	Chief	LB	NCI
Others:	S. Amsbaugh	Microbiologist	LB	NCI
	A.L. Burkhardt	Staff Fellow	LB	NCI
	J. Doniger	Senior Staff Fellow	LB	NCI
	N.C. Popescu	Microbiologist	LB	NCI
	S.S. Thorgeirsson	Chief	LEC	NCI
	P.J. Wirth	Expert	LEC	NCI
	S. Yasumoto	Visiting Associate	LB	NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Biology

SECTION

Somatic Cell Genetics Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
6.2	5.3	.9

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

To understand the nature of interrelationships between carcinogenesis and DNA metabolism, chromosome structure, and biological reagents, in vivo and in vitro approaches are being used. Although carcinogenic agents have a variety of similar deleterious effects on hamster and human cells, only hamster cells transform to the malignant state in a regular, predictable fashion. The control mechanisms involving proliferation and growth control are obviously different. Evidence linking proto-oncogene expression with specific chromosome translocations has been obtained from studies involving Burkitt's lymphoma and chronic myeloid leukemia. Carcinogen treatment of human foreskin fibroblasts may result in structural chromosome alterations which frequently involve proto-oncogene loci. However, no cells that induce progressively growing tumors have resulted. Therefore, additional genetic alterations are required for complete expression of malignancy with indefinite proliferation. Oncogenes are being studied utilizing hamster and guinea pig malignant cells. Malignant guinea pig cell lines, independent of carcinogen treatment, but not their preneoplastic progenitor cells contain an activated oncogene. Therefore, the acquisition of oncogene activation appears closely associated with tumorgenicity. Furthermore, the activated oncogene is of a different type hitherto described. A new model involving transfection of human papilloma (HPV) DNA into both human and rodent cells with cocarcinogens is under study. Human cells with the papillomavirus sequence have been obtained with a recombinant plasmid containing HPV 16. Transfection of this DNA into NIH 3T3 cells has resulted in a model for studying the molecular biology of viral DNA. Lastly, morphologic transformation and tumor production may be initiated by compounds which are not considered to be mutagens or to interact with DNA. Results with bisulfite indicate that qualitative polypeptide changes occur in the transition from the normal to the malignant state. Similar changes are seen as a result of malignancy induced by benzo-[a]-pyrene. Thus, a conversion of pathways responsible for carcinogenesis independent of the nature of initiation exists.

PHS 6040 (Rev. 1/84)

Project Description

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

Joseph A. DiPaolo	Chief	LB	NCI
S. Amsbaugh	Microbiologist	LB	NCI
A.L. Burkhardt	Staff Fellow	LB	NCI
J. Doniger	Senior Staff Fellow	LB	NCI
N.C. Popescu	Microbiologist	LB	NCI
S.S. Thorgeirsson	Chief	LEC	NCI
P.J. Wirth	Expert	LEC	NCI
S. Yasumoto	Visiting Associate	LB	NCI

Objectives:

The overall approach to problems in carcinogenesis is to investigate factors and mechanisms responsible for the modulation of neoplastic transformation of human and other cells, vital aspects in the etiology and prevention of cancer. Although cell biology was emphasized in the past, biochemical and molecular biological approaches are increasingly important in the elucidation of the objectives of the Somatic Cell Genetics Section. The specific objectives are (1) to define the role of chemical, physical, and biological agents pertinent to the process of carcinogenesis; (2) to characterize the cellular and chromosomal alterations associated with carcinogenesis; (3) to evaluate the relationships between DNA repair and metabolism and carcinogenesis; (4) to probe the somatic mutation aspects of experimental carcinogenesis.

Methods Employed: The "competence" of the target cells for transformation to the neoplastic stage differs from species to species. Therefore, the susceptibility to transformation is studied with cells from a variety of species: hamster, guinea pig, rat, and human. Each has its own advantage which ranges from quantitative morphologic transformation to defined stages of carcinogenesis and in the case of human cells, to the refractiveness of transformation. All procedures are performed with the view of understanding the underlying mechanism controlling the progression of the transformation phenomenon. Such an approach is required to determine whether or not the observed transformation is due to the direct or indirect effect of the carcinogen and in order to study the early events associated with neoplastic transformation. Cultures are made with freshly isolated cells from animals and humans or human cell strains obtained from patients with metabolic disorders that as controls have many of the attributes of "normal" cells, as well as cell lines which are known to exhibit some of the properties associated with nontransformed cells. Cells derived from whole embryos or specific organs are grown in medium with or without serum in the presence or absence of irradiated cells (feeder cells) and exposed to carcinogen transplacentally or prior to or subsequent to seeding the cells in plastic dishes. The transformation frequency takes into consideration the observed rate of transformation on a per cell basis or on the number of colonies obtained.

High resolution banding methods of prophase and prometaphase are used to identify structural chromosome alterations. Chromosome changes are further characterized by specific methods for visualization of constitutive heterochromatin (C band), ribosomal genes (N-band) and immunochemical methods using anti-nucleoside antibodies on denaturated chromosomes. The molecular in situ chromosome hybridization technique devised by us is being used for gene mapping and proto-oncogenes transposition on cancer cells.

DNA repair is measured by sucrose sedimentation, equilibrium density, and high-pressure liquid chromatography analysis of DNA extracted from carcinogen-treated cells. DNA replication is assessed by thymidine incorporation, DNA equilibrium density analysis, cytofluorographic analysis of cell cycle, cell autoradiography, and DNA fiber autoradiography.

Transformation of mammalian cells is accomplished by calcium phosphate precipitation, DEAE-dextan, protoplast fusion or eletrafusion. Analysis of DNA and RNA in transformed cells is by agarose gel electrophoresis, Southern blotting, DNA restriction analysis, gene cloning, c-DNA cloning, and RNA and DNA sequencing.

Major Findings:

Emphasis has continued to focus on the problems associated with obtaining transformed human cells utilizing Syrian hamster fetal cells (HFC). The use of cells from the two species is important because they permit comparison of a cell model that is readily transformable with one that is refractory to experimental transformation. Both the normal hamster and normal human have a stable karyotype and undergo senescence. We are concerned with why it is possible to obtain dose-dependent transformation of HFC that results in permanent neoplastic cell lines. No permanent malignant lines have resulted from treatment of normal human cells in vitro with a physical or chemical carcinogen. Experimental transformation of normal human fetal or foreskin cells as a result of carcinogenic insult (chemical, physical, or viral DNA) should be called "transitory" or intermediate transformation. Of the many properties of tumor cells, two of the most important are cell proliferation and loss of growth control. In fact, both at least are necessary for tumorigenicity. A cell that has lost only its growth control but does not continue to proliferate will not form a tumor because it will only divide for a finite number of generations before it senesces and dies. A cell that exhibits indefinite proliferation, but exhibits growth control will cease dividing when a signal of contact inhibition is activated. Therefore, new approaches are needed to study and to attain these critical characterizations of tumor cells.

A new approach for obtaining human cells susceptible to malignant transformation by chemical or physical carcinogens is to use a cancer promoting virus. Shope papilloma is known to work in concert with benzo[a]pyrene in cottontail rabbits and human papapillomas are associated with laryngeal and cervical carcinomas in humans. The laryngeal papillomas were converted to carcinomas by X-irradiation. The high incidence of human papilloma DNA sequences (HPV 16 or HPV 18) associated with cervical carcinoma but not condyloma acuminatum (cervical warts) suggests the HPV 16 (and/or 18) are involved in the initiation or maintenance of the malignant state. To understand how HPV 16 DNA is involved in the human

carcinogenic process, a recombinant plasmid and a neomycin (G418) resistant gene containing HPV 16 was transfected into human cells. Line HS27 transfected by an origin-defective SV-40 construct was the recipient; it is a non-tumorigenic cell line with indefinite proliferation. Several G418 resistant colonies were observed in the transfected culture, whereas none were found in control cultures. After selection, one half of each culture was treated with G418 continuously and the other half were each maintained in G418-free medium. After 10 days the cells were collected, the DNA was extracted, digested with Bam HI, and analyzed by Southern blot analysis for the presence of HPV 16 sequences. HPV 16 sequences were detected independent of maintenance in G418. Extra chromosomal DNA was also extracted from the cells and analyzed and HPV 16 sequences were found in all transfected cell strains.

HPV 16-transfected cells were X-irradiated (100 or 200 R) or treated with AcAAF (0.5 ug/ml). The treated cells were cultured until confluent and then seeded in agarose. Colonies (approximately 300 cells) were observed by 18 to 20 days and were isolated and seeded in G418 containing medium to observe the formation of neoplastic-like foci; these show criss-crossing growth, and overlapping and random orientation of cells. Foci were observed in AcAAF treated cells, but not in X-rayed or control cells. The tumorigenic potential of these foci are being analyzed. Similar experiments are being done with foreskin-derived fibroblasts and epithelial cells. G418-resistant fibroblasts have been obtained.

The biological significance of the role of HPVs in human cancer has not been elucidated. One major obstacle is the lack of any suitable <u>in vitro</u> model for analyzing the molecular biology of these viruses. To develop such a model, NIH 3T3 and C127 cells have been used as transfection recipients by HPV 16 DNA. A recombinant head to tail dimer of HPV 16 DNA (pSHPV16d) transformed NIH 3T3 cells after a long latent period (4 or more weeks). This is the first evidence that this putative etiological agent of cervical carcinoma is capable of transforming and can function biologically. It is unclear how the transforming activity of HPV 16 was established in the recipient cells because of the long latent period. To elucidate this, the physical state and gene expression of transfected HPV 16 DNA in recipient cells was analyzed. Recombinant DNA (pMHPV16d) carrying HPV 16 DNA and the neomycin resistant gene were transfected into NIH 3T3 cells. The cells were selected for resistance to the drug G418, a neomycin analog, a few days after transfection and a number of G418-resistant cell lines (MP3T3-G) were isolated within 1-2 weeks after transfection. G418-resistant cells exhibited a flat morphology similar to that of mock transfected NIH 3T3 cells. MP3T3-G cells possessed weak tumorigenicity; tumors developed after a long latent period (greater than 100 days) in only two of 9 innoculated nude mice. These results were comparable to the long latent period in the focus forming assay <u>in vitro</u> and indicate that HPV 16 DNA possesses oncogenic potential but does not transform by merely being present. A unique interaction between HPV 16 DNA and host cells relevant to the establishment of the fully transformed state appear necessary.

The response of NIH 3T3 cells to transfected HPV 16 DNA has two distinguishable phases: phase I, flat morphology with a higher saturation density (2-3 X 10^5 cells/cm^2) than that of mock transfected NIH 3T3 (1 X 10^5 cells/cm^2), but weak tumorigenicity (greater than 100 days); phase II, refractive, round cells with much higher saturation density (5-10 X 10^5 cells/cm^2) and highly

tumorigenic in nude mice (10 days). Next the physical state of the HPV 16 DNA in the recipient cells was analyzed. High molecular weight (HMr) chromosomal and extra chromosomal DNA were prepared from both phase I and phase II cells. Southern blot analysis of DNA from phase I cells indicated that HPV 16 in HMr DNA was extensively rearranged. Most rearranged forms in phase I were unstable suggesting the presence of extra chromosomal HPV 16 DNA. This was confirmed by Hirt extraction analysis. Phase II HPV 16 DNA demonstrated stable digestion patterns for several passages suggesting that HPV 16 DNA is stably associated with the host chromosome, probably existing in an integrated form. The recovery of extra chromosomal DNA was minimal from phase II cells. HPV 16 and HPV 18 DNA have also been found mostly as integrated forms in human cervical carcinoma; the current results suggest that integration is associated with the establishment of malignant transformation. The physical state of HPV 16 DNA is unique compared to other papilloma virus DNA such as bovine papillomavirus I, HPV I, and HPV 5 which exist as stable extra chromosomal DNA in transformed cells.

The genetic messages of HPV 16 in phase I and phase II cells were analyzed with respect to details of the genetic organization of HPV 16 genome (obtained from Seedorf and Rowekamp, personal communication). Poly A+ RNA was extracted from both phase I and phase II cells and subjected to Northern blot analysis with various fragments of HPV 16 DNA as probes. Phase I cells expressed five major poly A+ RNA species (5.8, 4.2, 2.5, 1.8 and 1.5 kb) and one minor band (3.5kb), whereas phase II cells expressed only 3 RNA species (4.5, 1.8, and 1.5 kb) in amounts less than those of phase I cells. The analysis with different restriction fragments, which represent a variety of putative genes, indicated that phase II cells expressed primarily early genes.

The role of early gene expression in the establishment of the transformed state is being studied. Together with the structural analysis of mRNA species, another approach is to alter early genes. The E6 gene which is considered to be one of the transforming genes for BPV 1 is of particular interest. Taq 1 cleaves once within HPV 16 DNA at a site close to the c-terminal end of the E6 gene. The recombinant pSHPV16d DNA was digested by Taq I yielding a full length linear HPV 16 DNA. The digested DNA was transfected into NIH 3T3 cells; morphologically transformed foci were obtained 6 weeks after transfection. Cells derived from the isolated foci were injected subcutaneously into nude mice; following inoculation, there was a long latent period (greater than 100 days) prior to development of tumors. Restriction endonuclease analysis of genomic DNA from these transformants demonstrated that HPV 16 DNA sequences were integrated. Furthermore, HPV 16 DNA in these cells lost the original Taq I recognition site. Thus, the lack of integrity of the E6 gene severely reduced the tumorigenic potential of the transformed cells.

Possible interactions between infected HPV 16 DNA and endogenous host genetic factors such as proto-oncogenes, tumor growth factors and their receptors, and putative growth control genes in normal cells may be necessary for transformation. Gene expression of several cellular proto-oncogenes in the phase I and phase II cells has also been studied. Poly A+ RNAs were subjected to Northern blot analysis and probed with v-myc, K-ras, v-fos, and v-src. The most significant quantitative differences occurred in myc gene expression. Compared to nontransformed NIH 3T3 cells, a 2 to 3-fold enhancement was detected in phase I and 5 to 7-fold in phase II cells; ras gene expression was enhanced approximately

2-fold in both phase I and phase II cells; and _fos_ gene expression was enhanced in phase I. A new RNA species (5.5 Kb) was induced in both phase I and II cells. _Src_-gene expression was not detected in NIH 3T3, phase I, nor phase II cells. These results suggest the importance of host factors in HPV 16 induced-transformation.

To understand the interaction of human proto-oncogenes with other genetic elements, the localization of these genes on human chromosomes is essential. Several proto-oncogenes have been molecularly cloned in the Laboratory of Cellular and Molecular Biology from a normal DNA library using viral oncogene probes to identify these sequences. Our new method for _in situ_ hybridization chromosome analysis was developed to localize DNA radiolabelled probes on human chromosomes. A transforming retrovirus of feline origin designated Gardner-Rasheed FeSV (GR-FeSV) consists of a Y-actin and tyrosine-specific protein kinase coding sequence designated v-_fgr_. By using a v-_fgr_ probe it was possible to isolate a human _fgr_ homolog which was distinct from the cellular homologs of other retrovirus oncogenes. For chromosomal localization, c-_fgr_ BE2 DNA was nick translated and H^3-DNA probes were used for _in situ_ hybridization on chromosomes obtained from methotrexate synchronized human lymphocytes. Grain distribution analysis on banded chromosomes resulted in the assignment of c-_fgr_ to the short arm of chromosome 1 at band 1p36.1. The results obtained with chromosomes derived from normal cells were confirmed by _in situ_ hybridization analysis of c-_fgr_ on an American Burkitt lymphoma cell line exhibiting a chromosome translocation between chromosomes 1 and 5 (t1;5,p36.1;q3) which involves the c-_fgr_ locus. In these cells the grains were located on the normal chromosome 1 as well as in the translocated segment of its short arm. The localization of the human _fgr_ proto-oncogene is of special interest because one of the two human _src_ loci was mapped on chromosome 1 at 1p34-36, a location coincidental with that of the human _fgr_ gene. Although _fgr_ and _src_ are distinct proto-oncogenes of the tryosine kinase gene family, these genes are closely linked and mapped in close proximity on human chromosome 1. The localization of human _fgr_ proto-oncogenes will also permit determination of structural alterations or whether expression of this gene is associated with changes involving chromosome 1. Such changes are characteristic for a variety of hematopoietic and solid tumors as well as human cells exposed _in vitro_ to chemical or physical carcinogens.

An important group of transforming genes are related to members of the _ras_ family of retroviral transforming genes. C-Ha-_ras_-1 and C-Ha-_ras_-2 are the homologs of the Harvey-murine sarcoma virus-transforming gene; c-Ki-_ras_-1 and c-Ki-_ras_-2 are the homologs of Kirsten-murine sarcoma virus. No viral counterpart has yet been isolated for N-_ras_. Previously with a panel of human-rodent somatic cell hybrids and filter hybridization techniques, c-Ki-_ras_ 1 and c-Ki-_ras_-2 were regionally assigned on chromosome 6 and the long arm of chromosome 12, respectively. _In situ_ hybridization analysis showed that c-Ki-_ras_-1 is located on 6p11-12 and c-Ki-_ras_-2 on 12p11.1-12.1 but not on the long arm of chromosome 12. N-_ras_ has been mapped to 1p11-13, bringing the number of proto-oncogenes located on chromosome 1 to five. Although chromosome deletions characteristic for neuroblastoma are distant from the N-ras-locus, the possibility remains that N-ras sequences may be altered or amplified within homogeneously stained regions (HSR) or double minutes (DM) frequently associated with this neoplasia.

Evidence linking proto-oncogene expression with specific chromosome translocations has been obtained from studies with Burkitt's lymphoma (BL) and chronic myeloid leukemia (CML). BL is a monoclonal B-cell derived malignancy with consistent and specific chromosome translocations. In the majority of BL cases the distal segment of the long arm of chromosome 8, (q24-qter), where the c-myc gene is located, is translocated to the distal end of chromosome 14(q32), the site of the immunoglobulin heavy chain locus. Two other translocations seen at lower frequencies occur between chromosome 8 and chromosomes 2 or 22 at either of the immunoglobulin light chain loci. A North American Burkitt lymphoma (NAB) was established from a tumor derived from a 14-year old female caucasian. The NAB-2 cell line has an aneuploid chromosome constitution with a modal chromosome number of 45. Although the cells were derived from a female patient, only one X chromosome was observed. The t(8;22)(q24;q11-12), reported to occur in approximately 20% of BL patients and involving the c-myc and lambda light chain genes, respectively, was observed in all NAB-2 cells examined. An interesting and unique chromosome alteration was observed on chromosome 2; the majority of NAB-2 cells have a structural abnormality on the short arm of this chromosome. On conventionally stained preparations the abnormality resembles an iso-gap or secondary constriction but with visible continuity of the chromosomal material. This alteration was observed on chromosomes prepared from methotrexate synchronized cells and logarithmetically growing cultures. The chromosome defect was localized to region 2p13-15 by G and R banding of metaphase and prometaphase chromosomes; a G-positive band not evident in the normal chromosome 2 at this location was present above the band 2p12 followed by a lightly stained region extending up to the band 2p15. The whole 2p13-15 region exhibited negative Giemsa staining on R banded chromosomes. The chromosomal defect involving 2p13-14 coincides with a constitutive fragile site identified on 2p13 and the break point in BL for the 2:8 translocation, but more importantly occurs at the site of the kappa light chain gene locus. It is possible that DNA or chromatin alterations associated with this visible chromosome defect may induce the secretion of immunoglobulin of Kappa type. This defect may also represent a chromosomal expression of gene amplification and conceivably could reflect the Epstein-Barr virus integration site.

The involvement of c-myc genes in BL is also well documented. The activation of c-myc appears to be due to its insertion into an immunoglobulin locus. C-myc is highly transcribed in NAB-2 cells. Alterations of c-myc structure or activity may confer a permanent loss of growth control which in turn may be responsible for the interaction of other proto-oncogenes during the process of tumor progression. In addition to an 8:22 translocation, NAB-2 cells have two other translocations, 1;5(p22;q33) and 3;7(pter-7q), not common for BL; these may be relevant to the neoplastic development of this line as the break points occur at the site of c-raf-1 and c-fms proto-oncogenes, respectively. Studies are in progress to determine whether the structure of the expression of c-raf-1 and c-fms have been altered as a result of these chromosome translocations. The analysis of NAB-2 cell lines revealed several unique and interesting characteristics relevant to carcinogenesis and tumorigenesis of BL--the presence of a specific and unusual chromosome defect at the locus of an immunoglobulin gene and the presence of chromosome translocations other than those common for BL involving proto-oncogene loci.

Chemical or physical carcinogen-induced DNA lesions involved in SCE formation might be the cause of proto-oncogenes alterations by a mutational event or by transposing, promoting, or enhancing sequences near transforming DNA sequences. Although a relationship between carcinogen induction of DNA adducts and SCE induction exists, only a subset of the total SCE-inducing lesions, those involving unequal exchange of DNA and persisting over several replicative cycles, may be important for the induction of neoplasia. Studies on the persistence of SCE and its relevance to the transformation process were extended using benzo(a)pyrene (BP), N-methyl-N'-nitrosourea (MNU), cis-platinum (II) diamine-dichloride (cis PtII), and methyl-methanesulfonate (MMS) on Syrian hamster fetal cells (HFC). A protocol utilized to examine the persistence of SCE and morphologic transformation consisted of 1 hr exposure to carcinogen followed by the addition of bromodeoxyuridine (BrdUrd) 1, 24, and 48 hr later. SCE were analyzed after two rounds of cell replication (24 hr) in the presence of BrdUrd. Morphologic transformation of colonies was examined six days after carcinogen treatment. BP (3µg/ml), cis-PtII (0.1µg/ml), MNU (50µg/ml), and MMS (40µ/ml) all induced a similar frequency of 50-60 SCE/cell when BrdUrd was added 1 hr after carcinogen. BP was the only carcinogen that caused an increased SCE frequency at 48 hr and persistent lesions at 72 as reflected by the SCE values. In contrast, lesions caused by MNU, cis PtII, and MMS were partially removed by 48 hr and completely removed by 72 h after treatment. A correlation between the persistence of damage generating SCE and the induction of transformation was observed for all these carcinogens. BP which caused the most lasting lesions also induced the highest transformation frequency, 3.3%. MNU, cis PtII, and MMS, whose lesions were completely removed within 72 h., caused similar low transformation frequencies of 0.6-0.9%. These results and those previously obtained with N-acetoxy-2-fluorenylacetamide, N-methyl-N'-nitro-N-nitrosoguanidine, mitomycin C, and ultraviolet light demonstrate that for a period of 48 hr after carcinogen insult, during which the cells undergo at least 4 replicative cycles, DNA damage generating SCE induced by all chemical carcinogens either persisted or was partially removed, whereas UV-induced lesions were completely removed. Although a consistent correlation between the persistence of SCE and the induction of transformation was not observed for all carcinogens, his study clearly illustrates that DNA damage generating SCE can persist over several replicative cycles, thus raising the possibility that lasting DNA alterations results in maximal induction of neoplastic cell transformation.

Intracisternal A-particles (IAPs) are retrovirus-like entities found in many mouse tumor cells, early mouse embryos, and occasionally in adult mouse tissues. Sequence elements homologous to IAP-associated high molecular weight RNA are reiterated about 1000-fold per genome in *Mus musculus*. Related sequences are found in the DNA of other rodents including the Syrian hamster. Individual IAP sequence elements ("IAP genes") cloned from the genome of mouse and Syrian hamster have structural properties characteristic of integrated retroviral proviruses. The distribution of IAP sequence over the Syrian hamster and mouse chromosome complement was analyzed using an *in situ* hybridization procedure. IAP-related sequences were associated with all of the Syrian hamster chromosomes but were clearly more concentrated in certain regions. This is particularly evident in the more heavily labelled karyotype where grains were densely clustered over the long arm of X, the short arms of 1, 3, 7, 8, 10, and 13, and the long arm of 20. The y chromosome was also the site of heavy labeling. All of these locations correspond to known regions of C-band positive late

replicating heterochromatin. There was no grain concentration over the heterochromatic short arm of chromosome 9. The short arm of chromosome 2 contains a small region of late replicating heterochromatin; although no dense grain cluster is apparent, IAP sequences were clearly concentrated in this chromosome arm. Centromeric and non-centromeric heterochromatin represent distinct categories of the Syrian hamster constitutive heterochromatin. The IAP sequences were preferentially localized in the non-centromeric heterochromatin. The grain distribution along the individual mouse chromosomes has shown that multiple IAP elements were associated with each chromosome and widely dispersed over the chromosome lengths. Certain prominent groups of grains were observed; e.g., in the distal portions of chromosomes 2 and 13 and the proximal half of 18, each of which must involve multiple IAP elements. The number of dispersed IAP genes copies per chromosome for the mouse and Syrian hamster and the relative sequence concentrations per chromosome length were estimated. Some chromosomes within each species appeared to be moderately enriched or depleted in IAP sequences. Overall, however, the data are consistent with a largely random partition of IAP sequences among the mouse chromosomes and the euchromatic chromosome arms of the Syrian hamster. The concentration of IAP sequences in the heterochromatic arms of Syrian hamster chromosomes is the first reported association of endogenous retroviral elements with vertebrate heterochromatin.

The HFC transformation model is an excellent, rapid, quantitative biassay that uses diploid cells and compares favorably with both experimental long-term animal studies and epidemiologic data. Similar to human cells, normal HFC senesce and spontaneous transformation is a rare event. Thus, the HFC model for transformation is relevant to the study of biology of carcinogenesis. Quantitative carcinogen-induced morphologic transformation is observed in a 7-day colony assay. The morphologic transformation frequency can be modulated so that the mechanism of factors that are responsible for enhancing or inhibiting transformation can be elucidated. Furthermore, *in vitro* morphologic transformation occurs in a dose-dependent manner and is characterized by random criss-crossing and piling up of cells not seen in controls; transformation correlates with tumorigenicity because individually transformed cell colonies can be isolated, cell lines developed, and the formation of tumors demonstrated by injecting the transformed cells into either neonatal Syrian hamsters or athymic nude mice. Furthermore, *in vitro* morphologic transformation is similar to that observed when primary tumors from *in vivo* hamster experiments are cultured.

Bisulfite is a food and pharmaceutical additive and a ubiquitous pollutant in the form of SO_2. Some epidemiological evidence indicates an association between SO_2 and cardiac and respiratory illness including lung cancer. Sodium bisulfite, a non-mutagen at neutral pH for both mammalian and bacterial cells, induces neoplastic transformation of HFC. Treatment of HFC with 5 to 20 mM bisulfite in phosphate buffer for 15 min results in dose-dependent transformation (up to 3%) without cytotoxicity. Colonies exhibiting altered morphology characteristic of transformation were isolated and seven cell lines developed. These cell lines exhibit anchorage independence in agarose; inoculation of 5 X 10^6 cells into nude mice (nu/nu) resulted in progressively growing fibrosarcomas. $NaHSO_3$ produced significant but minimal increases in sister chromatid exchanges (10 or 10 mM, 4/cell; 40 mM, 8/cell). By the second division after 40 mM $NaHSO_3$ chromosome aberrations occurred in only 4% of the cells. G-band analysis of 4 neoplastic cell lines revealed structural alterations and

aneuploidy. Two lines were aneuploid with mixed populations, one near diploid and tetraploid, and the other tetraploid and octoploid. The other two lines had abnormal chromosomes due to deletions, translocations, or centric fusions; one had a homogeneously stained chromosome. Because bisulfite induces no repair replication or nascent DNA strand changes, polypeptide expression was examined by 2 dimensional gel eletrophoresis (in collaboration with the Laboratory of Experimental Carcinogenesis). The gels were analyzed for both qualitative and quantitative polypeptide changes. Seven neoplastic lines all had the same qualitative changes. No change was observed either 0 or 48 h after sodium bisulfite. Polypeptides 1 and 2 (both pI 5.1; 52 and 28 kDa) were shifted slightly to the acidic side; polypeptides 3 (pI 6.8; 44 kDa) and 4 (pI 5.5; 46 kDa) were new; polypeptide 5 (pI 6.0; 55kDa) was missing. Similar qualitative changes were also found with a BP induced tumorigenic HFC line suggesting that these changes in gene expression are common to the transformation process. Line A (20-25 population doublings), after colony isolation, induced a tumor in only 1 of 12 nude mice, while the other 6 lines induced tumors in a minimum of 50% of the injected animals. After recloning in agarose, subline A1 induced tumors in 100% of the injected mice. In lines A, A1 and TA1 (tumor-derived) polypeptides 1 and 2 were shifted, polypeptide 3 was found and polypeptide 5 was missing. Polypeptide 4 was found in line A1 and TA1 but not in A. Therefore, the expression of polypeptide 4 appears associated with increased tumorgenicity. Early polypeptide changes were measured in a series of 20 sodium bisulfite-induced morphologic transformed colonies 2 days after isolation. Polypeptides 1 and 2 were always shifted and polypeptide 5 was missing. Polypeptide 3 was present in 7 of the colonies and polypeptide 4 was present in 14 of the colonies. Some colonies had neither 3 nor 4, both 3 and 4, or only 3 and 4. Thus polypeptide changes 1, 2 and 5 are all associated with early steps in the transformation process and related to the morphological changes, whereas polypeptides 3 and 4 appear to occur later and are more closely associated with the acquisition of tumorgenicity. Furthermore, 21 polypeptides were identified in the seven transformed lines that had consistent quantitative changes in expression. Ten of the proteins always increased and 11 always decreased. Thus it appears that there are coordinated changes in the expression of these polypeptides involved in the carcinogenic process. The qualitative polypeptide changes found in the bisulfite-induced malignant lines were similar to those seen in a benzo[a]pyrene-induced malignant lines. This suggests that there is a convergence of pathways responsible for the carcinogenesis independent of the nature of initiation.

Increased in vitro transformation frequencies resulting from TPA promotion can be inhibited by free radical scavengers; a phenomenon also observed in vivo with mouse skin painting experiments. The effects of superoxide dismutase (SOD) and Copper (II) 3,5-disoproplysalicylate (CuDIPS) on UV radiation induced transformation of HFC in vitro was studied. UV was used because it is not suspected to induce carcinogenesis by free radicals. Neither SOD nor CuDIPS, a SOD mimetic agent, effected transformation by UV alone. However, both compounds caused a dose dependent inhibition of TPA promoted UV transformation when given concurrently with TPA. No toxicity is attributable to either reducing agent. High molecular weight SOD acts at the cell surface, whereas CuDIPS penetrates throughout the cell. These results suggest that the superoxide anion radical (O_2) has a role in the promotion of transformation. Further experiments are required to precisely determine the role of free radicals in the biologic effects of TPA.

Although DNA repair and metabolism are accepted as relevant to carcinogenesis, the underlying processes are still obscure. Slow removal of O^6-methylguanine has been the basis for the hypothesis that this lesion is critical for carcinogenesis induced by methylating agents. For example, a positive correlation exists between the persistence of O^6-methylation in various tissues of the rat and their susceptibility to induction by N-methyl-nitrosourea (MNU) or dimethylnitrosamine, the brain being the most susceptible organ followed by the kidney and then the liver. The HFC system allows for a quantitative comparison of the induction and repair of methylated DNA lesions with the induction of transformation and lethality by a variety of methylating carcinogens. MNNG, MNU, or MMS concentrations that induce equivalent transformation frequencies in HFC, also induce similar levels of O^6-methylguanine but not in N^7-methylguanine. Therefore, these data are consistent with the concept that O^6-methylguanine is the lesion responsible for the initiation of carcinogenesis induced by methylating agents. Alkaline sucrose analysis of DNA from HFC treated with methylating carcinogens was used to study the effects of methylating agents on DNA metabolism with concentrations of MMS and MNNG that induced similar levels of O^6-methylguanine. Many more single strand (alkaline labile) breaks were observed in the DNA of HFC treated with MMS than with MNNG. The single strand breaks observed with MMS were induced by the presence of the N^7-methylguanine because there are 30-fold more of these lesions present in the cells treated with MMS than in those treated with MNNG. However, only one single strand break was observed for each 250 N^7-methylguanine; these probably represent incomplete repair of these lesions at the time of sampling. At a concentration of MNNG that induces one O^6-methylguanine per four replicons and one N^7-methylguanine per one-third of a replicon; no effect on the size distribution of nascent daughter strand DNA was observed even though the rate of DNA synthesis was inhibited by 20%. Furthermore, no effect on either chain elongation or replicon initiation could be detected in the size distribution of nascent strands. Therefore, a group of replicons exists that do not function in MNNG treated cells. Similarly, when a dose of MMS was used that gave equivalent levels of damage, again no observable effect on chain elongation or replicon initiation was obtained. However, the rate of DNA replication was inhibited by greater than 80%. These results indicate that the initiation of carcinogenesis by O^6-methylguanine is probably not mediated directly by effects on the rate of DNA replication and could likely be due to the miscoding properties of this lesion. One O^6-methylguanine is induced per 10^6 nucleotides when 1% of the HFC colonies are transformed; thus the target size of initiation of carcinogenesis is 10^4 nucleotides. Similar analysis of induction of mutation at the hgprt and ouabain loci by O^6-methylguanine indicates target sizes of 200 and 25, respectively. The larger target size for initiation of carcinogenesis suggest that either there are as many as 500 genes which can be mutated to initiate carcinogenesis or that simple base mutation is not the critical step for initiation.

Numerous transformed HFC cell lines have been established and characterized. To determine if carcinogen-induced transformation of HFC involved oncogene activation, high molecular weight DNA from various transformed cells was transfected into NIH 3T3 cells. Foci of morphologically transformed NIH 3T3 cells were observed after transfection with neoplastic HFC DNA, but not with DNA from non-treated normal HFC. To assure the activity of the putative hamster transforming sequence and to dilute out unlinked non-transforming sequences, DNA from several

of the transfectants was re-transfected into NIH 3T3 cells. Foci were scored and isolated. Cells derived from foci were selected for growth in soft agarose and expanded for further study. It was necessary to demonstrate that the morphological transformation of 3T3 cells observed after transfection was due to the uptake of the exogenous HFC DNA. When human DNA is transfected into NIH 3T3 cells, the highly repetitive human Alu sequence is used as a probe to demonstrate uptake of human DNA by transfected NIH 3T3 cells. To ascertain if transfected cells with neoplastic HFC DNA actually incorporated exogenous hamster DNA, total DNA from the transfected NIH 3T3 cell lines was screened for the presence of hamster intracisternal A particle (IAP) genes, a moderately repetitive sequence in HFC. Mouse and hamster IAP sequences can be distinguished by their Hind III restriction digest patterns. DNA from the transfected NIH 3T3 cells was digested with Hind II and subjected to Southern blot analysis with ^{32}P-labelled hamster IAP sequences as the probe. IAP sequences were detected in 5 of the 37 cell lines tested, indicating the uptake of hamster DNA by these cells. These 5 cell lines were selected for further analysis for the hamster transforming sequence. DNA from the transfected NIH 3T3 cell lines were screened with several known oncogene probes under relaxed conditions to establish if the putative hamster transforming sequence demonstrating activity in the NIH 3T3 assay was related to these known oncogenes. DNA from the transfected lines, neoplastic HFC lines, and non-treated HFC and NIH 3T3 cells was digested with restriction endonucleases Bam HI, Eco RI, or Hind III. The restricted DNA was subject to Southern blot analysis with ^{32}P-labelled H-ras, K-ras, bas, myc, fos, mos, src, or abl under relaxed conditions (30% formamide, 42°C). Where proto-oncogene homologs were detected in genomic HFC DNA, none of the above hamster proto-oncogenes were detected in the DNA of the NIH 3T3 transformed lines. In one case, hamster sequences were detected in the DNA of a second round transformed foci. This suggests that the IAP sequence is closely linked to the activated oncogene. The IAP sequence could be used as a probe to isolate this hamster-activated oncogene.

Cells at distinct preneoplastic stages of carcinogenesis have been identified and isolated in an in vitro guinea pig transformation system. These cells are being utilized for studying various aspects of transformation leading to malignancy and are particularly appropriate for analysis of oncogene activation. Five independent tumorigenic cell lines from carcinogen-treated guinea pig cells (initiated with 4 different carcinogens either in vitro or in utero) contain activated oncogenes. Because all of the oncogenes have weak homology to H-ras they were thought to have been derived from the same guinea pig proto-oncogene. Moreover, in all cases examined only tumorigenic cells, but not their preneoplastic progenitor cells, contain the activated oncogene. This suggests that oncogene activation was closely associated with acquisition of tumorigenicity. A 16 kb Bam HI DNA fragment containing the activated oncogene has been cloned from an MNNG transformed guinea pig cell line, 107C3. This cloned fragment transforms NIH 3T3 cells with high efficiency (1000 foci/μg); furthermore it can be detected in second round transformants of NIH 3T3 cells. The 16 kb fragment contains 3 interspersed subfragments with weak homology with H-ras. Moreover, Poly A+ RNA isolated from the guinea pig transformed line contains RNA messenger sequences of 3.6, 4.2, 4.4, and 4.6 kb which are homologous to the cloned activated oncogene. In contrast, the same poly A+ RNA contains a 1.2 kb species homologous to H-ras; 1.2 kb is the typical size of the H-ras transcript detected

in other mammalian species including rat, mouse, and man. These results suggest that even though the 107C3 activated oncogene has some homology with H-ras, it represents a different type of activated oncogene.

Significance to Biomedical Research and the Program of the Institute:

The prevention of cancer in humans depends to a large extent on understanding the process that is responsible for the development of transformation and on removing potentially harmful environmental agents. It is the aim of this project to investigate the refractiveness of human cells and to convert to the neoplastic state by human carcinogens. Epidemiologic evidence indicates that a number of carcinogens are capable of augmenting the cancer incidence; however, only rarely are "normal" human cells converted to the malignant state after carcinogen exposure. Furthermore, certain genes which become activated appear responsible for the expression of the neoplastic phenotype. The exact process by which the relevant genes become activated is not clear, nor are the pivotal stages known. This research also addresses these problems by attempting to identify the critical stages of carcinogenesis and to understand the underlying DNA and chromosome changes in order to find methods for intervening with the process or for preventing it.

Proposed Course:

In this project we will establish conditions and methods for *in vitro* transformation by chemical and physical carcinogens to determine the underlying biochemical and molecular processes responsible for the somatic cell changes which result in malignancy. The project will continue to define the conditions necessary for the quantitative transformation of mammalian cells with specific emphasis on human cells. A relevant question concerns the difficulty of transforming human cells relative to the ease of transforming Syrian hamster cells. Human and hamster cells senesce and have stable karyotypes; but only hamster cells, after carcinogen treatment, exhibit dose-dependent transformation that results in permanent tumorigenic lines. Because the hamster transformation model is responsive to promoters and anticarcinogens, it will be used to study free radical formation and surface alterations during transformation. The mechanisms(s) involved in transformation are probably independent of cell type; therefore, various sources of human epithelial and fibroblasts will be used, including those obtainable from patients with genetic disorders and from fresh normal foreskin. The use of normal cells (from individuals not cancer prone) results in a transitory transformed state. Carcinogen-treated human cells eventually lose their transformed attributes and fail to evolve into permanent lines. Therefore, experiments to determine the changes that result in continuous proliferation are being initiated. The approaches will include transfection using plasmids which contain sequences that confer extended growth potential. Carcinogen insult would occur either prior to or after transfection. Other experiments will be concerned with decreasing chromosome stability. In addition, because of the success obtained with papillomaviruses and chemicals in animals, human cell transformed human papilloma viral DNA 16, that will be used in cocarcinogenesis protocols. The viral DNA may serve as a cocarcinogen in conjunction with known chemical and physical carcinogens. After determining the conditions necessary for increasing the susceptibility of primary human fibroblasts, epithelial cells, such as from foreskin, will be used

because of the possibility that the human papilloma may require an epithelial target cell for its action as it does under natural conditions. The HPV 16/NIH 3T3 model that has been developed will be utilized to determine the role of gene expression and DNA structure in the biological consequences on the host cell. The investigation of the role of DNA lesions caused by physical or chemical carcinogens at the chromosomal and molecular level will be continued to determine the critical events leading to oncogene activation. In human cancer certain oncogenes have been activated at the site of specific chromosome translocations. The identification of altered DNA sequences is being correlated with chromosome rearrangements in human transformed cells. This permits the evaluation of the role of oncogene activation in initiation and progression of neoplasia. Human cells will also be utilized to isolate and characterize DNA sequences responsible for carcinogenesis induced by chemical or physical agents. In addition, the activation of oncogenes in the hamster and guinea pig transformation models is under continuing investigation. The specific DNA sequence alteration responsible for the activation of the isolated guinea pig oncogene will be determined as will the specific sequences that are expressed. Furthermore, this oncogene effect on gene expression of other guinea pig protooncogenes will be studied after its transfection into preneoplastic and noncarcinogen-treated cells.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01CP04673-14 LB

PERIOD COVERED
October 1, 1984 to September 30, 1985

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)
The Immunobiology of Carcinogenesis

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P.I.:	C.H. Evans	Chief, Tumor Biology Section	LB NCI
Others:	S.A. Barnett	Visiting Fellow	LB NCI

COOPERATING UNITS (if any)
None

LAB/BRANCH
Laboratory of Biology

SECTION
Tumor Biology Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
4.0	2.0	2.0

CHECK APPROPRIATE BOX(ES)
☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Lymphokines, interleukins, and other immunological hormones, i.e., the secretory bioregulatory macromolecules of lymphocytes, macrophages, and other leukocytes are being studied to define their effective anticarcinogenic and tumor cell growth inhibitory activities. Leukoregulin, a newly isolated lymphokine, can prevent carcinogenesis and inhibit tumor cell growth. Anticarcinogenic action is direct, irreversible and occurs without cytotoxicity. Inhibition of tumor cell growth is primarily reversible but can become irreversible due to increased susceptibility of preneoplastic and neoplastic cells to cytolytic destruction by natural killer cells resulting from leukoregulin target cell interaction. Leukoregulin at very high concentrations is also directly cytolytic for tumor cells. The direct acting anticarcinogenic activity of leukoregulin is more potent than the tumor cell inhibitory activity; but by also being able to increase target cell sensitivity to the cytoreductive action of naturally cytotoxic lymphocytes, leukoregulin may be an effective homeostatic mechanism for control of carcinogenesis at its later stages of development. Leukoregulin anticarcinogenic, tumor cell growth inhibitory, and cytoreductive sensitizing activities, copurify and are distinct molecularly and biologically from interleukin I, interleukin II, lymphotoxin, macrophage migration inhibitory factor, macrophage activating factor, and interferon. Leukoregulin alters cell surface conformation, membrane fluidity and permeability, and membrane glycoprotein synthesis with changes in the latter correlating directly in time and quantity to leukoregulin-induced establishment of the anticarcinogenic state. Leukoregulin induces identical changes in target cells as are present during natural killer cell cytotoxicity providing strong evidence that it is an intrinsic mediator or element of the natural cytotoxicity reaction and possibly signifying its central role in immunological homeostasis.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

C.H. Evans	Chief, Tumor Biology Section	LB NCI
S. A. Barnett	Visiting Fellow	LB NCI

Objectives:

This project provides a means to study and understand the potential of the normal immune system to prevent, suppress, inhibit and even enhance the growth of an incipient tumor cell during carcinogenesis. Natural cytotoxicity of macrophages, lymphocytes and lymphokines alone or in combination are being studied at various stages of carcinogenesis to provide insight into the immunobiology of cancer. As the host mechanisms and the target cell structures with which the immune effectors interact are delineated, it will be possible to investigate how natural and induced immunity may be augmented to suppress and even prevent the final aspects of carcinogenesis--the transition from the preneoplastic to the neoplastic state.

The primary objective of this project is to elucidate at the target cell level the relationship between cell surface alterations accompanying the development of carcinogenesis and host mechanisms capable of preventing, otherwise inhibiting, or even enhancing the development of cancer. Specific objectives include (1) identification of somatic cell alterations during carcinogenesis using in vitro model systems to allow study of membrane and other phenotypic changes at specific steps or stages in carcinogenesis and (2) investigation of host interactions with specific cell surface alterations during carcinogenesis. Particular emphasis is placed on natural and induced cellular and humoral immunobiological interactions due to the frequent occurrence of neoantigens, re-expression of fetal antigens, and alterations in alloantigens on tumor cells.

Methods Employed:

Normal and malignant animal and human cells in culture including chemical and physical carcinogen-treated cells at progressive stages in the transformation process are studied for somatic cell changes such as altered morphology, morphological transformation, anchorage independent growth and tumorigenicity in relation to their interaction and response to components of the immune system. Immunobiological techniques including direct and indirect immunofluorescence, flow cytometry, complement fixation, colony inhibition, radionuclide uptake and release, delayed hypersensitivity skin reactions, and tumor transplantation rejection are employed in analyzing cell membrane changes and in assessing host interactions to the changes. A major emphasis is placed upon flow cytometry and cell sorting to identify plasma membrane and intracellular alterations responsible for regulation of cell proliferation and carcinogenesis.

Major Findings:

During the past several years we have demonstrated that lymphokine preparations prepared from antigen or mitogen stimulated lymphocytes contain a potent anti-carcinogenic activity which is the property of a new lymphokine termed leukoregulin. Leukoregulin is able to prevent as well as inhibit at early and late stages the subsequent development of radiation or chemical carcinogenesis. The anticarcinogenic state does not affect cell proliferation and like many responses to hormones is induced rapidly and persists for a short time. When the leukoregulin anticarcinogenic state is present at the time of carcinogen exposure the further development of initiated or complete carcinogenesis is irreversibly inhibited. In the later stages of carcinogenesis the anti-cancer action of leukoregulin is characterized by inhibition of cellular proliferation which in fully tumorigenic cells at high lymphokine concentrations may also be accompanied by cellular destruction. An additional action of leukoregulin is its ability to increase the sensitivity of cells to destruction by natural killer cell cytotoxicity. These bioactivities--prevention of transformation, inhibition of neoplastically transformed cell growth, and augmentation of the sensitivity to natural killer cell cytotoxicity--co-purify during sequential isoelectric focusing, ion exchange and molecular sizing chromatography purification of the lymphokine. Leukoregulin, furthermore, is separated by these methods from the interleukin, lymphotoxin, and interferon activities present in the unfractionated lymphokine preparation.

Leukoregulin induces specific target cell plasma membrane changes accompanying its natural killer cell sensitizing and tumor cell proliferation inhibitory activities. The membrane changes can be rapidly detected by flow cytometric analysis of both light scatter and membrane permeability changes, the latter being followed by the uptake or by the loss of intracellular fluorescent molecules such as fluorescein or propidium iodide, respectively. The same changes are observed in target cells during the course of natural killer cell cytotoxicity. This suggests that leukoregulin may be an intrinsic mediator or element of natural lymphoid cell cytotoxicity and occupy a central role in immunological homeostasis.

Cells in all phases of the cell cycle are sensitive to the proliferation inhibitory action of leukoregulin. Flow cytometric analysis of human K562 erythroleukemia cells treated with increasing concentrations of leukoregulin to inhibit more than 90 percent of the cell replication demonstrates that the percentage of cells in each phase of the cell cycle remains constant during 72 hours of leukoregulin treatment. Fluorescence-activated cell sorting of leukoregulin plasma membrane permeability altered cells also reveals that cells in each phase of the cell cycle are susceptible to the membrane perturbing action of leukoregulin. Flow cytometric analysis of the sorted cells, moreover, indicates that cells in the "S" phase of the cell cycle may be more sensitive to the acute membrane destabilizing action of leukoregulin. This cell cycle independent pattern of inhibition of cell replication and its rapid reversibility are quite different from the cell cycle phase specific blocks observed with the typical inhibitors of cell replication affecting protein and/or nucleic acid synthesis.

The molecular events underlying the perturbation in plasma membrane stability and permeability following target cell interaction with leukoregulin have been studied by contrasting the changes in leukoregulin-treated cells with those after exposure of target cells to a variety of membrane active agents affecting ion transport. Compounds including oubain, amphoteracin B, calcium ionophores A23187 and X-537A, phospholipase C and A2, and phytohemagglutinin which increase intracellular calcium levels mimic the membrane changes induced by leukoregulin. Calmodulin, calcium channel blockers and both sodium and potassium ionophores and channel blockers exhibit no flow cytometrically detectable membrane destabilizing activity. The calcium stimulators are active over a wide range from 10^{-3} to 10^{-10}M and the one paralleling the kinetic activity of leukoregulin most closely is the calcium ionophore A23187. This suggests that leukoregulin may exert its anti-cancer action in part by altering intracellular calcium levels. Furthermore, measurement of intracellular ionic calcium using the calcium binding fluorescent probe quin2 reveals a transient increase in intracellular calcium approximately 10 minutes after treatment of human K562 erythroleukemia cells with leukoregulin. Treatment of the cells with calcium ionophore A23187, however, produces an increase in intracellular calcium within one minute of ionophore exposure indicating that although leukoregulin may alter calcium levels, it does not function solely as an ionophore. Newer and more specific fluorescent calcium indicators are being used to further define the bioactivity of leukoregulin and other lymphokines in terms of calcium metabolism and membrane stability and their relationship to preventing carcinogenesis and inhibiting the proliferation of neoplastically transformed cells.

Significance to Biomedical Research and the Program of the Institute:

This investigation provides the first evidence that a single lymphokine possesses the ability to directly prevent as well as inhibit the further development of chemical or radiation carcinogenesis at several distinct stages during the transition from a normal to a neoplastic cell. The evidence, moreover, indicates that leukoregulin functions in concert with lymphoid cells as an intrinsic element or mediator within natural lymphoid cell immunoregulatory actions and as an extrinsic immunological anticarcinogenic hormone. Moreover, identification that the biological activities are accompanied by rapid and transient biochemical events at the plasma membrane opens new avenues to explore the pathways regulating cell proliferation and transformation. Leukoregulin has a very high degree of specificity in terms of its ability to prevent transformation and to inhibit proliferation of transformed cells. It may be this specificity that regulates the early events in reactions culminating in membrane cytotoxicity and control of abnormal cells. This investigation provides fundamental new insights into normal anticarcinogenic immunological mechanisms increasing both our understanding of homeostasis and basic physiological mechanisms influencing the development of carcinogenesis.

Proposed Course:

Investigations will continue to define the mechanisms whereby neoplastically transformed mammalian cells acquire the susceptibility to cytotoxic activity of naturally immune cellular and humoral effectors. In particular the biochemical events responsible for the membrane destabilizing activity of leukoregulin in

relationship to the anticarcinogenic and cell proliferation inhibitory actions of the lymphokine will be further defined. A major question still to be resolved is the relationship of leukoregulin to lymphotoxin and the structurally related macrophage hormone, tumor necrosis factor, and to interferon. Each of these immunological hormones directly alters target cell membrane stability. In some instances their actions are synergistic and in others antagonistic. Continuing developments in flow cytometry will allow further resolution of the relationships among the anti-cancer actions of these lymphokines. Definition of these relationships will clarify both the role of immunological hormones in the development of carcinogenesis as well as increase our understanding of target cell pathways regulating normal cellular homeostasis and the transition to neoplasia.

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ANNUAL REPORT OF

THE LABORATORY OF CELLULAR CARCINOGENESIS AND TUMOR PROMOTION

NATIONAL CANCER INSTITUTE

October 1, 1984 through September 30, 1985

The Laboratory of Cellular Carcinogenesis and Tumor Promotion plans, develops and implements a comprehensive research program to determine the molecular and biological changes which occur at the cellular and tissue levels during the process of carcinogenesis. Studies are designed to (1) define normal regulatory mechanisms for cellular growth and differentiation; (2) determine the mechanism by which carcinogens alter normal regulation and the biological nature of these alterations; (3) investigate the mechanism by which tumor promoters enhance the expression of carcinogen-induced alterations; (4) identify cellular determinants for enhanced susceptibility or resistance to carcinogens and tumor promoters; (5) elucidate the mechanism by which certain pharmacologic agents inhibit carcinogenesis.

The Laboratory is composed of three sections each of which is charged with a major responsibility for portions of the Laboratory goals. Because of the integrated approach toward an understanding of mechanisms of carcinogenesis, considerable interaction occurs among the sections. Areas of interaction are defined in the individual project reports.

IN VITRO PATHOGENESIS SECTION: The In Vitro Pathogenesis (IVP) Section (1) develops relevant model systems for the study of all phases of the process of carcinogenesis; (2) defines regulatory mechanisms for the normal control of growth and differentiation and alterations in these controls induced by initiators and promoters; (3) produces, isolates and studies initiated cells; (4) studies functional alterations in gene expression produced by initiators and promoters and the mechanism by which these functional changes occur; (5) elucidates factors which determine susceptibility to carcinogenesis.

This section has directed its efforts toward both developing in vitro model systems to study chemical carcinogenesis in epithelial cells and to use these systems to study the mechanisms of tumor initiation and promotion. Mouse epidermis, the classic model for induction of squamous cancer by chemicals, has been adapted for in vitro study. Previous investigations had demonstrated that this model is a close in vitro analogue of the mouse skin carcinogenesis system in vivo. In vitro, epidermal cells proliferate and differentiate, metabolize carcinogens, repair DNA damage, and respond to tumor promoters like epidermis in vivo.

Regulation of Epidermal Growth and Differentiation: Previous results from this Laboratory have indicated that extracellular calcium concentration regulates epidermal proliferation and differentiation. Culture medium of 0.02 - 0.09 mM calcium concentration selects for proliferating cells which have morphological, immunological and biochemical characteristics of basal cells. Culture medium of >0.1 mM induces epidermal differentiation resulting in cessation of proliferation, vertical stratification, cornification and sloughing of mature squames.

The regulation of differentiation by calcium is not associated with changes in cyclic nucleotide levels but appears dependent on a functioning Na^{+}-K^{+} ATPase. There are strong similarities in the induction of epidermal differentiation by calcium and by the tumor promoter, 12-O-tetradecanoylphorbol-13-acetate (TPA). Both alter protein synthesis and phosphorylation in a common pattern as evidenced by computer analysis of polyacrylamide gel electrophoresis patterns of radiolabeled proteins. TPA-induced differentiation is likely to be mediated through activation of protein kinase C, and calcium may also mediate differentiation via phospholipid metabolism and protein kinase C activation since phosphatidylinositol metabolism is rapidly and specifically activated when basal cells are placed in high calcium medium. Retinoids can inhibit the induction of differentiation by both TPA and calcium. Retinoids are potent inducers of a tissue transglutaminase activity which is not normally expressed in skin. Retinoids also inhibit cornified envelope formation by both Ca^{++} and TPA. Epidermal differentiation is associated with a reduction in synthesis of several unidentified basal cell proteins of 10, 11 and 12 kd. Desmosomal proteins are modified during epidermal differentiation.

Quantitative Assay for Carcinogen-induced Altered Differentiation: The capability to selectively grow basal cells in low calcium medium and induce differentiation in high calcium has provided an assay to select for cells with altered differentiative responses. Exposures of primary cultures of mouse keratinocytes to chemical carcinogens results in foci which resist the Ca^{++} signal to differentiate and continue to proliferate under high Ca^{++} conditions, producing countable colonies which stain red with rhodamine B. Cells obtained from mouse skin initiated in vivo show the same characteristics. A number of characteristics of these foci as well as the characteristics of their induction suggest they are initiated cells. Twenty-seven cell lines have been derived from these foci and their properties compared to 6 cell lines derived from chemically induced mouse papillomas. The two cell types were similar in many respects particularly in their capacity to grow in high Ca^{++} medium and their resistance to TPA induced terminal differentiation. Both cell types failed to yield transforming genes when their DNAs were transfected into NIH 3T3 cells. However, transfection of an activated ras^{Ha} oncogene into papilloma cells resulted in an altered phenotype. Transfected cells were highly malignant when transplanted into nude mice.

Infection of epidermal cells with oncogenic retroviruses containing an activated ras gene indicate that expression of ras and subsequent synthesis of p21 provides a marked proliferative stimulus to basal cells. However, such cells respond to high Ca^{++} by cessation of proliferation. These cells do not terminally differentiate but appear to be blocked in some non-terminal state of differentiation. Interestingly, blocked cells appear to remain responsive to tumor promoters and do not synthesize pemphigus antigen, a marker of suprabasal differentiation in vivo. Exposure to TPA reactivates their proliferative activity. Thus an activated ras gene could serve to produce a conditionally initiated cell which could only display the tumorigenic phenotype after exposure to a tumor promoter.

Mechanism of Action of Tumor Promoters and Antipromoters: Tumor promotion by phorbol esters has been an area of intense study in this Section. Many aspects of skin tumor promotion suggest that cell selection plays an important role in the process. Our studies have indicated that basal cells are heterogeneous in response to phorbol esters in that some cells are induced to differentiate while others are stimulated to proliferate. This could form the cellular basis for selection. The molecular basis for the pharmacological heterogeneity is suggested by studies of the phorbol ester receptor in cultured keratinocytes. Multiple receptor classes are found in differentiating cultures indicating that maturation state may modify receptor affinity. Protein kinase C activation may be the major pathway which mediates phorbol ester responses in keratinocytes since exogenous diacylglycerols can mimic the effects of TPA. Furthermore, generation of endogenous diacylglycerols by exposure of cells to phospholipase C reproduces the biological effects of TPA. A consequence of phorbol ester induced differentiation is the production of single strand DNA breaks as measured by alkaline elution. This effect is blocked by retinoic acid and does not occur in initiated cells which are resistant to TPA-induced differentiation. Interestingly, benzoyl peroxide, another skin tumor promoter, produces DNA strand breaks rapidly and directly, without inducing differentiation and some initiated cell lines are also resistant to this effect of benzoyl peroxide. This differential effect of benzoyl peroxide on normal and initiated cells could form the basis for cell selection in tumor promotion by that agent.

While pursuing mechanistic studies on tumor promotion *in vitro*, skin painting experiments are conducted *in vivo* to define the biology of promotion. Phorbol ester tumor promoters are incapable of accelerating conversion from benign to malignant tumors. However, inhibitors of papilloma formation prevent carcinoma development suggesting a precursor relationship. Three protocols have been developed which yield papillomas with a high risk for conversion. The first stage of tumor promotion can be accomplished by time alone and no exogenous exposure is required. Together these results indicate that tumor promotion involves cell selection and clonal expansion of initiated cells but does not alter their preneoplastic character. A second genetic change is required in the conversion of benign to malignant lesions.

Immunological Techniques to Study the Interaction of Carcinogens with DNA: Antibodies specific for carcinogen-DNA adducts have probed the nature, extent, and consequences of *in vitro* and *in vivo* DNA modification. DNAs substituted with 2-acetylaminofluorene (AAF), benzo[a]pyrene (BP), or *cis*-diamimine-dichloroplatinum II (*cis*-DDP) were analyzed by quantitative immunoassays able to detect one adduct in one hundred million nucleotides, and by immunohistochemical procedures developed to localize adducts *in situ*. In hepatic DNA of rats fed a carcinogenic dose of AAF for 4 weeks, adduct accumulation reached a plateau at 2-3 weeks and adducts were shown by immunohistochemistry to be primarily localized in the periportal areas. During 4 subsequent weeks on control diet, adduct removal was biphasic. A computer-derived pharmacokinetic model consistent with this data proposed that adducts are formed into two genomic compartments, one from which adducts are removed rapidly and another from which they are removed slowly. In contrast to the high levels of AAF adducts formed in rat liver DNA, at least 50-fold lower adduct quantities were formed in the DNA of mouse epidermis and cultured mouse epidermal cells exposed to initiating doses of BP. When activated forms of both carcinogens were utilized in the keratinocyte

focus assay, N-acetoxy-AAF yielded more adducts per molar concentration than the BP derivative but no differentiation-altered foci formed in N-acetoxy-AAF treated cultures. Nucleated peripheral blood cell DNA was obtained from cancer patients at multiple times during courses of cis-DDP therapy, and a total of 223 samples were analyzed. Of these, 23 untreated control samples were negative, and 46% of the 200 samples from patients receiving cis-DDP were positive. Adduct accumulation, in positive patients, occurred as a function of total cumulative dose, suggesting relatively slow adduct removal. Disease response data on 47 patients indicated that individuals with adduct levels greater than 200 attomoles/μg DNA have a very high (65%) rate of complete response to therapy. Parallel experiments in animal models have demonstrated that the same adduct forms in kidney, gonads, and tumors of rats and mice in direct relation to dose.

Determinants for Susceptibility to Carcinogenesis: Epidemiological and medical genetic data have indicated major individual differences in cancer risk in humans. Increased risks are associated both with overall susceptibility to cancer or susceptibility in a particular target organ. In some cases specific genetic changes have been associated with increased risk, but in many examples polygenic influences appear more likely. To date biochemical epidemiological studies have focused only on genetic differences in carcinogen metabolism. In the complex and multistage evolution of cancer, it seems unlikely that carcinogen metabolism is solely responsible for enhanced risks. In fact, it seems likely that factors associated with the expression of neoplastic change would play an important role in host susceptibility. The development through selective breeding of animal strains with high susceptibility at a particular organ site provides an excellent model for the study of susceptibility determinants. This Laboratory has utilized the SENCAR mouse for susceptibility studies since this stock is especially sensitive to chemically-induced skin carcinogenesis.

SENCAR mice are markedly susceptible to two-stage skin carcinogenesis compared to BALB/c mice. Grafting studies have shown that susceptibility is a property of the skin itself and other studies indicate that sensitivity is not due to differences in metabolism of polycyclic aromatic hydrocarbons. Yet by a variety of biological and biochemical parameters SENCAR epidermal cells behave identically to epidermal cells from less sensitive strains. These include in vitro growth kinetics, DNA repair, receptor binding of growth factors and phorbol esters, density and function of Langerhans cells, production of epidermal thymocyte-activating factor, and induction of transglutaminase.

Data from both in vivo carcinogenesis experiments and in vitro focus induction assays after carcinogen exposure suggest the existence of a constitutively initiated cell population in SENCAR epidermis, but the role of such cells in susceptibility has not been clearly defined. Carcinogen-induced epidermal cell foci in vitro which resist terminal differentiation induced by Ca^{++} appear to be initiated cells. Although the number of foci arising from SENCAR cells and the more resistant Balb/c cells are similar in response to equivalent carcinogen treatments, such foci are qualitatively different from cells of the susceptible strain Furthermore, SENCAR cell lines derived from these foci respond differently biochemically than Balb/c lines. Most notably, TPA treatment of SENCAR lines leads to much greater induction of the proliferative enzyme ornithine decarboxylase. Recent in vivo experiments have also shown

that after attaining a peak incidence, SENCAR papillomas regress even when tumor promotion is continued, while in the more resistant CD-1 mouse, papilloma incidence continues to increase in the presence of tumor promoters but declines in their absence. Carcinoma incidence is the same in both cases. These results imply that promoter-dependent papillomas are not on the pathway to malignancy. Several lines of evidence suggest that responses to promoters may play a major role in SENCAR susceptibility in addition to the evidence that the nature of the initiated cells may differ between strains.

Molecular Regulation of Epidermal Specific Differentiation Products: Our studies have indicated that initiation of carcinogenesis is associated with a change in normal differentiation. In order to understand this association at the molecular level, the regulation of specific differentiation products is being explored. Keratin peptides are a family of proteins which comprise the major cytoskeletal and differentiation molecules of epidermis. We have cloned the genes which code for the major keratins of proliferating and differentiating keratinocytes. Their expression has been studied in keratinizing vaginal epithelium where a hormone-regulated, coordinated, sequential program related to proliferation and differentiation has been determined. Interestingly, the expression of these genes is markedly altered in skin treated with TPA and in carcinomas. These results are consistent with other evidence for a fundamental derangement of differentiation in promoter-treated epidermis and in malignant epidermal cells. On the basis of this expression data, we have developed an assay using antisera that are monospecific for individual keratin subunits that can distinguish between benign and malignant tumors. Sequence analyses of keratin cDNAs have revealed unique structural aspects of keratins expressed at different differentiation-states that may change the properties and functions of the filaments that they form. Isolation of a genomic fragment of one of the differentiation keratins has been completed and the gene structure has been characterized by sequence analysis in comparison to the cDNA.

DIFFERENTIATION CONTROL SECTION: The Differentiation Control Section (1) studies the biological and biochemical factors involved in normal differentiation of epithelial tissues; (2) uses pharmacological techniques to alter differentiation of normal, preneoplastic and neoplastic epithelial cells to determine the relevance of differentiation to carcinogenesis and to determine methods to intervene in preneoplastic progression; (3) studies the relationship between differentiation and growth control; (4) focuses on cell surface changes in differentiation and neoplasia.

Vitamin A and its derivatives, the retinoids, are of interest in cancer research because they play an essential role in the maintenance of normal differentiation in most epithelial tissues, under normal physiological conditions. At the biochemical level our laboratory has demonstrated that retinol and retinoic acid regulate membrane glycosylation reactions. Such biochemical involvement is consistent with the reported alterations in glycosylation of α_{2U}-globulin and α_1-macroglobulin in vitamin A-deficient livers, and it might explain altered oligosaccharide composition of fibronectin secreted by chick sternal chondrocytes cultured in excess retinoic acid.

Vitamin A as a Regulator of the Dolichylphosphate-mediated Pathway of Protein Glycosylation: Work from our laboratory has demonstrated that the condition of vitamin A deficiency causes a marked (up to 95%) decrease in the incorporation

of mannose into glycoproteins in vivo. Accompanying this decrease in protein mannosylation were an accumulation of free mannose, and a decrease in guanosine diphosphatemannose and in dolichylphosphate mannose in severely deficient livers in vivo. Reduced food intake was shown to be primarily responsible for the depletion of guanosine diphosphate mannose in severely vitamin A-deficient hamsters (6 weeks on a vitamin A-deficient diet). Recent data demonstrate that accumulation of dolichylphosphate occurs in hamsters kept for only 4 weeks on a vitamin A-depleted diet, i.e., much before any other symptoms of deficiency become manifest. In these animals liver guanosine diphosphate mannose levels were above normal, while the amount of free mannose and of mannose incorporated into dolichylphosphate mannose, oligosaccharide lipids and glycoproteins was within normal ranges. Therefore, accumulation of unglycosylated dolichylphosphate seems to be the earliest measured effect of vitamin A deficiency and it likely explains the profound affect of the vitamin on glycosylation reactions.

A new mannolipid was found and characterized as phosphatidylmannose. This compound is synthesized from phosphatidic acid and guanosine diphosphomannose in liver microsomal membranes and the quantity found is directly dependent on vitamin A status of the animal.

Expression of Keratin Polypeptides in Tracheal Metaplasia Associated with Vitamin A Deficiency and Carcinogen Exposure: Vitamin A is essential for the normal growth and differentiation of epithelial cells. In tracheobronchial epithelium, normally a mucus secreting tissue, deficiency of vitamin A causes squamous metaplasia. This lesion is similar to that observed in vivo in tracheas treated with benzo[a]pyrene and occurs in organ cultures of tracheas obtained from hamsters kept for four weeks on a vitamin A deficient diet and cultured in a vitamin A-depleted, chemically-defined medium. Retinoids added to the medium can prevent the development of the lesions and restore mucociliary differentiation. It was of interest to identify and characterize keratin subunits in vitamin A-deficient tracheas, as part of our laboratory's effort to understand the mode of action of vitamin A in normal epithelial differentation and its involvement in anticarcinogenesis. In the present studies, we have employed a tracheal organ culture system and have reproduced the in vivo phenomenon of squamous metaplasia during culturing under vitamin A-free conditions as well as after carcinogen treatment. The squamous metaplasia induced by vitamin A deficiency, both in vivo and in vitro, was accompanied by an overall increase in keratin synthesis. Vitamin A-deficient tracheas were shown by immunoblot analysis to contain keratins of 50, 48, 46.5 Kd detected with the antibody AE_1, and 58, 56 and 52 Kd detected with AE_3. These proteins were either absent or present in much less quantity in control tracheas. In deficient tracheas 60 Kd keratin was found to be located specifically in squamous suprabasal cells, and 55 and 50 Kd keratin proteins were found in a greatly expanded basal cell compartment. Following carcinogen exposure, the appearance of 60 Kd keratin and the enhanced expression of 50 and 55 Kd keratins preceded the squamoid metaplastic response as detected morphologically. Both the keratin changes and the morphological changes were prevented by retinoid treatment.

Studies on Retinoid Transport: Plasma and intracellular retinoid binding proteins have been isolated by several investigators. Although their precise function is unknown, they may mediate the transport of retinol, retinoic acid and possibly other retinoids from tissue to tissue or within cellular and

intracellular compartments. We have developed an assay which permits the simultaneous quantitation of cellular retinol and retinoic acid binding proteins by high performance liquid chromatography on columns of Mono Q. Such an assay will greatly facilitate our studies on the function of the retinoid binding proteins.

MOLECULAR MECHANISMS OF TUMOR PROMOTION SECTION: Using relevant model systems, the Molecular Mechanisms of Tumor Promotion Section 1) studies the interaction of tumor promoters with specific cellular receptors, 2) elucidates the functional importance of receptors in promoter action, 3) identifies endogenous ligands with specific affinity for receptors of exogenous promoters, 4) characterizes endogenous factors mediating receptor affinity and response, and 5) clarifies the initial biochemical steps in the cascades associated with receptor occupancy. Understanding of the early events in promoter action should permit the analysis of their control, modulation, and function in human cells under normal and pathological conditions. Determination of the ability of less specific tumor promoters to perturb indirectly the same processes will shed light on the generality of mechanisms of promotion and will assist in the development of better assays for tumor promoters.

Phorbol Ester Receptor Purification and Biochemistry: Both our evidence and that of others strongly argue that protein kinase C is the major phorbol ester receptor. An impediment to biochemical and immunological analysis of the receptor has been that the published purification protocols are time consuming, afford low yields (0.5-5%), and are difficult to scale up. Taking advantage of new advances in column chromatography, we have now developed purification protocols that permit the rapid and efficient isolation of the receptor. We have also identified stabilization procedures to preserve receptor activity, which is otherwise quite labile once the receptor is in the purified state. The purified receptor has been used for preparation of polyclonal antibodies, which are suitable for immunoprecipitation and Western blot analysis. Efforts are on-going to prepare monoclonal antibodies.

Characterization of the purified receptor indicates that it possesses separate domains for enzymatic and binding activity. The properties of the binding fragment are being determined. The availability of the purified receptors permits the analysis of substrates for the kinase and demonstration of its role in biological processes. Particular interest has been focussed on the possible role of active oxygen species in phorbol ester action. In collaboration with Dr. A. I. Tauber, we have been able to demonstrate that protein kinase C can reconstitute the oxidative burst using the neutrophil γ-membrane subfraction.

Interaction of Phorbol Ester Receptors with Phospholipids and Lipophilic Ligands: We had previously demonstrated that the phorbol ester binding, like the kinase, showed an absolute requirement for phospholipids, i.e., the binding protein is actually an apo-receptor and phospholipids are an essential cofactor. We have now characterized, in detail, the phospholipid requirements for binding, with particular emphasis on the contribution which the phospholipid makes to the properties of the complex as a whole. We find that different phospholipids play a major role in determining the binding characteristics of the receptor. Not only do they determine the specific binding affinity of [^{3}H]phorbol 12,13-dibutyrate ([^{3}H]PDBu) for the receptor but they are also capable of determining

the structure-activity relations for binding. Our current model is that localization of protein kinase C into different lipid environments may help account for the evidence from both biological and binding experiments that indicates receptor heterogeneity.

The high evolutionary conservation of the phorbol ester receptor had suggested the existence of an endogenous phorbol ester analog. Based on the postulated physiological role of protein kinase C, diacylglycerol derivatives appeared to be attractive candidates for the endogenous analog. We have demonstrated that diacylglycerols indeed inhibit phorbol ester binding in a competitive fashion. Furthermore, under appropriate experimental conditions, the ratio of diacylglycerol to receptor approaches 1:1, again consistent with competitive binding. Comparison of the relative binding affinities of phorbol and glycerol derivatives with homologous side chains indicates that the phorbol esters show moderate to considerably greater affinity, depending on the specific side chain.

The in vitro studies with diacylglycerols suggested that the endogenous generation of diacylglycerols in cells by addition of phospholipase C or the exogenous addition of diacylglycerols (of the appropriate lipophilicity to transfer from the aqueous media to the cells) would similarly affect phorbol ester binding and induce similar responses to the phorbol esters.

The effects of phospholipase C treatment were tested in two systems, mouse keratinocytes (collaborative experiments with Drs. Lichti and Strickland, IVP, LCCTP) and rat pituitary cells. In both systems, phorbol ester responses were mimicked, diacylglycerol generation was confirmed, and phorbol ester binding was competitively inhibited, as predicted. In the latter system, moreover, similar receptor translocation to membranes was found as had previously been observed in response to phorbol ester treatment. The second approach for elevating cellular diacylglycerol levels was to expose the cells to exogenously added diacylglycerol. In NIH 3T3 cells, treatment with GMA (glycerol 1-myristate 2-acetate), like PMA, inhibited EGF binding and stimulated phosphatidylcholine turnover. Both responses were more transient with GMA than with PMA, suggesting rapid breakdown of the GMA. In mouse keratinocytes, likewise, dicaproin, dicaprylin, and OAG (1-oleoyl 2-acetylglycerol) inhibited binding of EGF and induced ornithine decarboxylase and transglutaminase. Responses were less than those seen for PMA, again consistent with instability of the diglycerides.

An important consequence of the identification of protein kinase C as the phorbol ester receptor is that it provides the opportunity to determine whether other tumor promoters may also affect it. At high concentrations, unsaturated fatty acids have been reported to be tumor promoters. We found that unsaturated fatty acids both stimulated protein kinase C enzymatic activity and inhibited phorbol ester binding. The inhibition of binding appeared to reflect a mixed mechanism. Several other, structurally unrelated classes of tumor promoters had no effect on protein kinase C activity.

A related objective has been to clarify phorbol ester structure-activity relations in an effort to design antagonists to the phorbol esters. As part of that effort, we have begun a collaborative effort with an organic synthesis

group at Standford to determine the binding activity of structural variants in the phorbol ester backbone as well as of compounds very different from the phorbol esters in structure but possessing similarities in the three-dimensional positioning of postulated critical functional groups. Both approaches have yielded compounds with weak but measurable activity.

Analysis of Phorbol Ester Binding: Considerable data suggest heterogeneity of biological responses to the phorbol esters in mouse skin. Similarly, we have obtained evidence for three classes of binding sites in mouse skin particulate preparations which differ in their binding affinities and structure-activity relations. To further explore the functioning of the phorbol ester receptors in the biologically relevant system of mouse skin, we have characterized the binding of phorbol esters to intact mouse keratinocytes in collaborative studies with the IVP section. We find that mouse keratinocytes also display heterogeneous binding, consistent with two classes of binding sites. Upon Ca^{++} induced differentiation, the total level of phorbol ester binding increases, apparently due to an increase in the amount of the lower affinity site. Several Ca^{2+}-resistant cell lines have been developed by LCCTP from carcinogen-treated primary keratinocyte cultures. [^{3}H]PDBu binds to these cells at a single site, and the binding site has characteristics similar to the higher affinity site of the normal keratinocytes in low calcium medium. We conclude that the state of epidermal differentiation can modulate the amount of the lower affinity binding site for the phorbol esters. Current efforts are directed at determining whether the different sites are coupled to different biological responses.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP04504-13 CCTP

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Model Systems for the Study of Chemical Carcinogenesis at the Cellular Level

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	S. H. Yuspa	Chief	LCCTP	NCI
Others:	H. Hennings	Rssearch Chemist	LCCTP	NCI
	M. Poirier	Research Chemist	LCCTP	NCI
	D. Roop	Senior Staff Fellow	LCCTP	NCI
	J. Strickland	Research Chemist	LCCTP	NCI
	U. Lichti	Expert	LCCTP	NCI
	J. Harper	Staff Fellow	LCCTP	NCI

COOPERATING UNITS (if any)

Univ. of Washington, Seattle, WA (Karen Holbrook); Microbiological Assoc., Bethesda, MD (E. F. Spangler); Princeton Univ., Princeton, NJ (M. Steinberg); Medical College of Wisconsin, Milwaukee, WI (Akihoro Kusumi)

LAB/BRANCH

Laboratory of Cellular Carcinogenesis and Tumor Promotion

SECTION

In Vitro Pathogenesis Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
9.0	5.0	4.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Cellular and molecular aspects of chemical carcinogenesis in lining epithelia are studied in mouse epidermis by in vivo and in vitro techniques. Normal epidermal growth and differentiation are regulated by extracellular calcium. Calcium may exert its effects indirectly via regulation of intracellular concentrations of sodium and potassium. This control appears to involve phosphatidylinositol turnover and generation of diacylglycerol which activates protein kinase C. Carcinogens alter the regulation of epidermal differentiation. This change is highly correlated to the initiating event in carcinogenesis. Altered cells have a number of characteristics similar to papilloma cells. An activated ras oncogene also alters epidermal differentiation, but this effect is conditional and may be modulated by exposure to a tumor promoter. Initiated cells or mouse epidermal tumors do not transcribe unusually high levels of ras or other known retroviral oncogenes. However, transfection of an activated ras gene into papilloma cells causes them to become highly malignant and the tumors are anaplastic. Phorbol ester tumor promoters accelerate epidermal differentiation, and this can be blocked by antipromoting retinoids which induce a unique transglutaminase possibly counteracting the transglutaminase induced by promoters. DNA strand breaks are produced as a consequence of phorbol ester mediated terminal differentiation and these are blocked by retinoids. While initiation and promotion both involve changes in epidermal differentiation, another genetic change is required prior to carcinoma formation. This change can be accomplished by genotoxic agents but not by tumor promoters. Only a subclass of papillomas are at risk to progress to carcinomas.

PHS 6040 (Rev. 1/84)

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

S. Yuspa	Chief	LCCTP	NCI
H. Hennings	Research Chemist	LCCTP	NCI
M. Poirier	Research Chemist	LCCTP	NCI
D. Roop	Senior Staff Fellow	LCCTP	NCI
J. Strickland	Research Chemist	LCCTP	NCI
U. Lichti	Expert	LCCTP	NCI
L. De Luca	Research Chemist	LCCTP	NCI
J. Harper	Staff Fellow	LCCTP	NCI
A. Jeng	Expert	LCCTP	NCI
P. Blumberg	Research Chemist	LCCTP	NCI
S. Jaken	Expert	LCCTP	NCI
S. Thorgeirsson	Chief	LEC	NCI
J. Stanley	Senior Scientist	DB	NCI

Objectives:

To study cellular and molecular changes during stages of chemical carcinogenesis through the development and use of cultures of epithelial lining cells which are the major target site for cancer in humans. Studies are directed to give insight into general changes occurring in specialized mammalian cells during malignant transformation and specific molecular events which may be causative to the transformation process. Specific markers of the the transformed phenotype of epithelium are also being sought and mechanisms to prevent or reverse transformation are being studied.

Methods Employed:

This laboratory has developed and utilized mouse epidermal cell culture as an appropriate model to approach the stated objectives. Previous studies have shown that this model functions biologically in a fashion highly analogous to mouse skin *in vivo*. Human epidermal cells obtained from neonatal foreskins have also been adapted to growth *in vitro*. *In vivo* studies utilizing the initiation-promotion model for mouse skin carcinogenesis and grafts of human or mouse skin onto nude mice are also employed. A number of laboratory techniques are required to pursue the objectives. Morphology is followed by light and electron microscopy and histochemical staining. Macromolecular synthesis and growth kinetics are studied by biochemical and autoradiographic procedures and flow cytometry. Intracellular ion changes are assayed by atomic absorption spectrometry. Cellular metabolic functions, including the production of specific differentiation products, are monitored by enzyme assays, one- and two-dimensional gel electrophoresis, amino acid analysis, and radioimmunoassay. Protein purification techniques employ column chromatography, fast protein liquid chromatography, and high pressure liquid chromatography. The progression to the malignant phenotype is monitored by growth rates, soft agar assay, karyotypic abnormalities, enzymatic changes, changes in gene expression at the level of mRNA and injection of cells into nude or newborn mice.

A number of immunologic techniques including monoclonal and polyclonal antibody production, fluorescent staining, immunoblotting, immunoprecipitation, and radioimmunoassay are being performed to recognize the normal or altered phenotype and to study specific molecules. Isolation of specific mammalian genes is performed through the preparation of epidermal mRNA, reverse transcription, and cloning of transcripts in plasmid pBr322 and the screening of genomic libraries. Cloned genes are characterized by hybridization-selection assays, blot analysis and sequencing. DNA transfection is utilized to isolate transforming gene sequences.

Major Findings:

The pursuit of this project has led to major new findings in four pertinent areas: 1) factors controlling normal epithelial differentiation; 2) quantitation, selection, and characterizaton of carcinogen-altered epidermal cells; 3) biochemical and molecular genetic characterization of specific marker molecules and assessment of their regulation in normal and transformed epidermal cells; 4) understanding of the process of preneoplastic progression and the mechanisms of tumor promotion and anti-promotion.

Much of the progress in this project has developed from the discovery that ionic calcium is a critical regulator of epidermal growth and differentiation. At low ionic calcium concentrations in culture medium (0.02 - 0.09 mM), epidermal cells maintain a monolayer growth pattern with a high proliferation rate. Such cells do not form desmosomal attachments. Essentially 100% of the attached cells are in the proliferating cell pool. Our studies have shown that these cultures have low transglutaminase activity, virtually no cornified cells in the substrate-attached population and synthesize the bullous pemphigoid antigen, all three characteristics of basal cells. The pemphigus vulgaris antigen, a marker for epidermal cells in a more advanced state of differentiation, is not expressed.

When cells maintained under low calcium growth conditions are switched to medium with calcium content above 0.1 mM (standard commercial culture media are 1.2 - 1.8 mM), differentiation is induced. Desmosomes form within minutes of exposure to high Ca^{++} medium. These cells vertically stratify, form cornified cell envelopes and slough from the culture dish. This program of differentiation is characterized by a high transglutaminase activity and the synthesis of the pemphigus vulgaris antigen. The synthesis of bullous pemphigoid antigen ceases during Ca^{++}-induced differentiation. Following the addition of Ca^{++} to culture medium, DNA synthesis, as measured by thymidine incorporation or autoradiography, remains unchanged for 10 hours and then decreases rapidly to less than 10% of control by 24 hours and less than 5% by 48 hours.

Earlier studies from this laboratory had indicated that retinoids could modulate epidermal differentiation. We have proposed that this effect of retinoids is important in their anticarcinogenic activity. We were surprised to discover that retinoids were potent inducers of a transglutaminase (TGAse) activity in epidermal cells. Epidermal transglutaminase is responsible for cornified envelope (CE) cross-linking during terminal differentiation. Our studies also indicated that retinoids were inhibitors of envelope formation which could be stimulated by Ca^{++} or the tumor promoter 12-O-tetradecanoyl phorbol-13-acetate (TPA). Both Ca^{++} and TPA induce epidermal transglutaminase. The retinoid-reduced TGAse is distinct from the epidermal TGAse by a variety of enzymatic

properties. On the basis of immunological cross-reactivity, the retinoid enzyme appears to be identical to the tissue TGAse which is not associated with CE production. Retinoic acid completely inhibits the increase in epidermal TGAse-induced by TPA but reduces the induction by Ca^{++} only slightly. Therefore, it is unlikely that retinoic acid inhibits CE formation only by its effect on epidermal TGAse. A mechanism for inhibition of CE formation involving the action of this enzyme on cornified envelope precursors before their proper assembly is still being considered, although it appears now less likely, since under some culture conditions (high cell density, high Ca^{++}) that favor CE formation, retinoids induce little transglutimase activity but block cornification. Furthermore, simultaneous treatment of cells with RA and TPA greatly reduces the induction of tissue TGAse. Nevertheless, in vivo the tissue TGAse is rapidly induced (maximum by 4 h) by a single application of 10 nmoles of RA to adult mouse skin and the enzyme activity remains elevated for at least 72 h. Application of TPA in vivo simultaneously with RA or 4 h later leads to greatly reduced levels of tissue TGAse by 24 h suggesting a more rapid turnover of this enzyme in TPA-treated tissue.

Whereas the induction of TGAse in vivo is sustained, frequent additions of RA to the medium of cultured cells is required to maintain high TGAse activity. In collaborative studies with the Differentiation Control Section of LCCTP, the metabolism of RA in epidermal cell culture has been studied. ^{14}C-Labeled RA is rapidly metabolized in culture. The half-life of RA in the medium is about 10 h, and metabolism requires the presence of cells.

Methods to detect and quantitate CE precursors are essential before the effect of RA on their synthesis, assembly and cross-linking can be assessed. CE precursor proteins from mouse epidermis have not previously been characterized. Antibody to intact CE was prepared in rabbits and shown to react with 65-70 kd proteins from epidermis; however, these proteins do not appear to accumulate in cultured primary epidermal cells under conditions where CE are formed.

The regulation of epidermal differentiation by Ca^{++} in cell culture suggested that a calcium-binding protein might play a physiological role in epidermal differentiation in vivo. Several years ago in France a 13 kd protein was isolated from rat skin and described as a skin Ca^{++}-binding protein (SCaBP). Antibodies supplied to us from France immunoprecipitated antigens of 10, 11, and 12 kd from [^{35}S]-methionine-labeled cultured epidermal cells. The synthesis of these antigens was regulated by Ca^{++} or TPA. The 11 kd antigen was a phosphoprotein and served as a substrate for protein kinase C in vitro. Attempts to purify SCaBP from newborn mouse epidermis failed to yield copurification of antigenic and Ca^{++}-binding activity. Studies using an antiserum to muscle paralbumin (PV) revealed identity of PV and SCaBP by immunoblotting. Immunoblots and immunohistochemistry performed on rat skin components showed that the 13 kd PV/SCaBP antigen was located in the panniculus carnosus, a muscular component of the dermis, while the 10, 11, and 12 kd antigens were epidermal antigens. The epidermal antigens are likely the same as those modulated by Ca^{++} and TPA in cultured basal cells, hence efforts are underway to purify them and produce antisera and monoclonal antibodies specific for them. In addition, screening of a phage lambda gt_{11} expression library of newborn epidermis cDNA with SCaBP antiserum has yielded a clone expressing high levels of antigen. Efforts to characterize the sequence in the clone coding for the antigenic protein are

underway. These results indicate that SCaBP is not a basal epidermis cell marker protein but rather is PV, located in the dermis. Our attention has refocused on the previously unidentified epidermal antigens whose synthesis is regulated by differentiation.

Studies to characterize differentiation markers in mouse keratinocytes have included an examination of the synthesis of desmosomal components and their assembly. Desmosomes form within minutes of switching cultured keratinocytes from low to high Ca^{++} medium. In collaboration with Akihiro Kusumi of the Medical College of Wisconsin and Malcolm Steinberg of Princeton University, we have studied, at the protein level, the basis for appearance of desmosomes. Immunoprecipitation of desmosomal proteins metabolically-labeled by [^{35}S]-methionine in low and high Ca^{++} cells has revealed that the Ca^{++} environment modulates the relative rates of synthesis of three desmosomal protein families. In addition, the 240, 210, and 150 kd desmosomal proteins are all phosphorylated in culture in both high and low Ca^{++} medium and are substrates for protein kinase C _in vitro_. These studies may be useful for identifying regulatory processes in the control of desmosome formation during differentiation and could provide markers for altered differentiation or altered adhesion in the skin.

The mechanism by which Ca^{++} induces terminal differentiation in keratinocytes has been under study for several years. We have reported that intracellular ion changes appear to be regulatory in the process. In recent published studies we have suggested that both Ca^{++} and TPA may induce epidermal differentiation via a common pathway involving protein kinase C, the phorbol ester receptor. In collaboration, with Dr. Sue Jaken of the Molecular Mechanisms of Tumor Promotion Section of LCCTP, the metabolism of phosphotidylinositol (PI) has been examined. There is a rapid increase in PI turnover shortly after epidermal basal cells are exposed to increased Ca^{++} in the culture medium. This is manifested by an increase in the metabolites IP_1 and IP_3, a rapid decrease in PI and an increase in phosphatidic acid. These changes occur within a few minutes and are consistent with an action for Ca^{++} which leads to the generation of diacylglycerol and activation of protein kinase C.

In collaboration with Drs. Peter Wirth and Snorri Thorgiersson of LEC, we have computer analyzed 2-D gel electrophoresis patterns of newly synthesized proteins from cells treated with Ca^{++} or TPA. From 600-1200 spots are visible on autoradiographs of proteins prepared from cultured mouse epidermal cells labeled with a ^{14}C-amino acid mixture and separated in two dimensions over a pH range of 5-8 and a molecular weight range of 10-130 kd. The rate of synthesis of 11 proteins were altered by both Ca^{++} and TPA within 1-4 hours; 7 were increased and 4 were decreased, with changes in the same direction for both efffectors. Additional studies involved an analysis of protein phosphorylation changes common to both inducers. At least one protein of molecular weight 42 kd is phosphorylated to a greater extent within 30 minutes of exposure to either Ca^{++} or TPA. The results suggest that a common program of differentiation is induced by both Ca^{++} and TPA.

The emphasis we have placed on understanding the regulation of normal epidermal differentiation evolves from our discovery that carcinogen exposure _in vivo_ or _in vitro_ yields keratinocytes whose response to differentiation signals is

altered. In the cell culture model where Ca^{++} induces differentiation, such cells are selectable since they produce expanding colonies under high Ca^{++} growth conditions. A number of characteristics of this model suggest that the cells which are selected are initiated cells. We have derived 27 cell lines from differentiation-altered foci for further analysis. Some lines were derived from initiation in vivo and subsequent selection in culture, while others were derived by in vitro treatment and selection. The lines were not tumorigenic nor did they grow in agar. Most were either 2N or 4N in DNA content and were negative for gamma glutamyltranspeptidase activity. All synthesized keratin proteins, but the pattern was similar to that of normal keratinocytes. All lines grew well in high Ca^{++} medium and were resistant to the induction of differentiation by TPA.

In order to compare the characteristics of these lines to those expected of papilloma cells, we established conditions to isolate viable cells from mouse skin papillomas induced by initiation and promotion of SENCAR mice. Viable cells were plated onto dishes coated with collagen-fibronectin-albumin in dermal fibroblast conditioned medium with 0.05 mM Ca^{++} and 10 ng/ml EGF. Six cell lines were established from separate primary isolates and designated PA, PB, PC, PD, PE, PF. At clonal density, all lines grew better in low Ca^{++} medium than in high Ca^{++} medium. When switched to high Ca^{++} medium, 4 of the 6 lines produced large numbers of cornified envelopes. In these respects, the lines differed from putative initiated cell lines derived from carcinogen exposure of primary keratinocyte cultures. Exposure to TPA stimulated the growth of all lines and did not induce differentiation in any. This latter characteristic was similar to findings for presumed initiated cells. Retinoic acid inhibited growth of all lines. The pattern of keratins synthesized by each line was identical to that of normal cultured keratinocytes in low or high Ca^{++} medium. Of interest was the finding of high expression (5-10 fold) of the fos oncogene compared to normal cells, whereas the expression of transcripts for ras^{Ha}, ras^{Ki}, myc, fes, raf, and abl was similar to normal controls. PA and PE produced squamous cell carcinomas in some recipient mice, while the other lines have not been tumorigenic.

In order to understand genetic determinants of the initiated phenotype, we have been studying the characteristics of keratinocytes infected with oncogenic retroviruses. Infection of mouse basal keratinocytes with Harvey or Kirsten sarcoma virus enhances the proliferation rate and consequent cell number three- to five-fold. However, virus infected cells respond to the differentiation signal of high Ca^{++} medium by growth arrest. These cells do not progress through their entire maturation program, but instead remain as a confluent, quiescent monolayer of mature keratinocytes with high transglutaminase activity but a low rate of cornified envelope formation. Virus infected cells in high Ca^{++} medium synthesize a unique 58 kd protein in cytoskeletal extracts which is not synthesized in virus infected cells in low Ca^{++} medium or in control cells in either medium. Studies with temperature-sensitive mutants have shown that all of the virus effects are dependent on a functioning viral ras gene and that p21 expression is not modulated by the state of epidermal differentiation.

Closer analysis of virus infected keratinocytes in high Ca^{++} medium reveals that they synthesize the pemphigoid antigen but not the pemphigus antigen, and they can return to their basal cell morphology when placed in low Ca^{++} medium. Both of these findings are consistent with these cells being in a late stage

of basal cell maturation. Unlike normal cells in high Ca^{++} medium, virus infected cells respond to TPA by an induction of ornithine decarboxylase activity, a reduction in transglutaminase activity and a stimulation of [^{3}H]thymidine incorporation. These results suggest that an activated ras gene in keratinocytes causes a conditional change in cell maturation which alone is insufficient to achieve the initiated phenotype. However, exposure to TPA complements the ras phenotype and allows expression of a cell type consistent with the biology of a papilloma.

In order to further explore the role of oncogenes in epidermal carcinogenesis, a series of experiments involving mRNA expression or DNA transfection have been performed. The expression of ras^{Ha}, ras^{Ki}, fos, myc, and raf mRNA in papilloma and carcinoma tissue derived by a variety of chemical carcinogenesis protocols appeared to be similar to that of normal murine epidermis by slot blot analysis. In contrast, fos expression was significantly elevated in five of six cell lines established from papillomas. Repeated exposure of mouse skin to various tumor promoters and hyperplastic agents resulted in a decrease in abl expression. Since abl and fos are both strictly regulated during early murine embryogenesis, our findings further support the notion that these genes may play a critical role in cell proliferation and differentiation.

DNA isolated from each of the papilloma cell lines (PA-PF) was transfected into NIH-3T3 cells in order to detect mutated or otherwise activated transforming genes. All of the papilloma DNAs failed to morphologically transform 3T3 cells. However, transfection of the T24 bladder oncogene (mutated ras^{Ha}) into each papilloma cell line has resulted in a significant enhancement of the clonal growth characteristics of papilloma cell lines in high Ca^{++} medium. Furthermore, these lines become highly tumorigenic and the recipients develop rapidly growing anaplastic carcinomas which are lethal after several weeks. This implies that papilloma cells may express an oncogene, other than those already tested, that contributes to tumor progression by acting in cooperation with an activated ras^{Ha} gene. This is of particular interest in view of recent published reports that ras^{Ha} activation may be of ultimate significance in chemical carcinogenesis.

Further development of a transfection system involving primary epidermal cells and papilloma cell lines as DNA recipients will allow us to directly examine the role(s) of abl and fos in epidermal differentiation, as well as to detect new genes that may act in cohort to alter differentiation patterns and ultimately contribute to tumor progression.

Many aspects of skin tumor promotion suggest that cell selection plays an important role in the process. Previous studies from this laboratory have provided a cellular basis for selection. Subpopulations of epidermal basal cells respond heterogeneously to phorbol ester tumor promoters in that some cells are induced to differentiate while others are stimulated to proliferate. Additional studies have indicated that more mature basal cells respond in the differentiative pathway. In another section of this report, the lack of a differentiative response for putative initiated cells and papilloma cells was described. Thus, promotion could be based on selective normal cell loss and clonal expansion of initiated cells. A consequence of phorbol ester-induced differentiation in keratinocytes

is the production of single strand DNA breaks as measured by alkaline elution. This effect is blocked by retinoic acid, which also inhibits phorbol ester-induced differentiation; chymostatin, fluocinolone acetonide, catalase and superoxide dismutase do not block strand breaks or differentiation. Initiated cell lines resistant to the induction of differentiation do not form strand breaks in response to phorbol esters. Benzoyl peroxide rapidly induces DNA strand breaks in keratinocytes but does not induce differentiation. Some cell lines resistant to the induction of differentiation and strand breaks by phorbol esters are sensitive to strand breaks by benzoyl peroxide. However, some initiated cell lines show cross-resistance to strand breaks and cytotoxicity by benzoyl peroxide. This resistance could be the basis for cell selection during promotion by benzoyl peroxide.

In vivo, phorbol esters also appear to accelerate differentiation of maturing keratinocytes. Cells are rapidly lost from the differentiating compartment after mouse skin is exposed to phorbol esters since mRNA transcripts for differentiation-associated keratins rapidly decrease when epidermal RNA is probed with cDNAs for these genes. Taken together, these results indicate that phorbol esters produce an imbalance in epidermal homeostasis due to accelerated loss of one subpopulation and selective growth in another. Our results further predict that initiated cells form a compartment which is resistant to the differentiative influences of phorbol esters or the cytotoxic effects of benzoyl peroxide. This compartment would selectively expand in cell number with each promoter exposure to ultimately yield a benign tumor.

While pursuing mechanistic studies on epidermal carcinogenesis in vitro, in vivo carcinogenesis experimentation is actively pursued under contract N01-CP1-5744. Data obtained previously have indicated a three-stage requirement for carcinoma formation after skin painting. Initiation appears to require a genotoxic carcinogen while promotion by phorbol esters results in a large number of benign tumors. Continuous promoter exposure does not influence the carcinoma yield but subsequent exposure of papilloma-bearing mice to genotoxic agents markedly accelerates and enhances carcinoma yield (malignant conversion). Recent in vivo studies have been useful to further define the biology of tumor induction by inhibitors and promoters.

When promotion of papilloma formation is operationally divided into two stages, Stage 1 is accomplished equally well by TPA or by a 10-week delay in the start of Stage 2 mezerein treatment. After this delay, mezerein is an effective tumor promoter. Thus, the mechanisms involved in the two operational stages of promotion may not differ. The complete reversibility of Stage 1 TPA promotion emphasizes the likely epigenetic mechanism of promotion of papilloma formation. When papilloma formation is inhibited by antipromoters, a similar reduction in carcinoma incidence occurs, suggesting that a papilloma stage is required as a precursor to carcinomas. Three new treatment protocols have been defined which yield papillomas with a high probability for malignant conversion. Furthermore, protocols have been designed for chemical treatment of papilloma-bearing mice which can modulate the metastatic pattern of the carcinomas which evolve.

Significance to Biomedical Research and the Program of the Institute:

The majority of human cancers are associated with environmental exposures, and most of the tumors are of epithelial origin. The development of a cell culture model system for epithelial carcinogenesis has been a major requirement for understanding the specific cellular and molecular alterations associated with malignant change in these specialized cells. The epidermal cell culture system has provided a required model. In previous years we have demonstrated a strong parallel in the biologies of epidermis in vitro and in vivo. Our current studies have focused on control mechanisms for normal growth and differentiation, on alterations produced by initiators and promoters, and on markers associated with the transformed phenotype. We have discovered a primary regulation of epidermal differentiation in our calcium studies. Analogous work has subsequently been pursued in other laboratories and it now appears that calcium is an important regulator of differentiation in esophagus, bronchus, bladder and mammary epithelium. Cell culture model systems for these other important epithelial target organs which share characteristics of our model are being developed in other laboratories. Our current studies in this project are defining the biochemical pathways crucial for differentiation to proceed normally in epidermis. Presumably parallels will exist in a variety of models. The importance of understanding normal differentiation, aside from its inherent value, is emphasized by our findings that an early event in chemical carcinogenesis is an alteration in differentiation control. We have used this alteration to develop an assay system capable of categorizing potency of initiators, a first for epithelial cells, and for primary cultures. Furthermore, we have isolated a number of preneoplastic cell lines and have sought markers to characterize their altered biology. The definition of such markers could be useful for recognizing preneoplastic cells in vivo and thereby facilitating early diagnosis and chemoprevention efforts. The availability of models in which discrete changes can be induced by carcinogens, in which cell lines can be followed for progressive transformed phenotype, and in which conversion to malignancy can be monitored provide valuable resources to analyze the role of oncogenes in tumorigenesis. Experimentally, tumor promotion is the major influence in determining latency period and an important factor in determining target site for carcinogenesis. It is likely that the promotion phase has a similar importance in human carcinogenesis. Our studies have provided a biological basis for tumor promotion in skin, and the pharmacological mechanisms involved are becoming clarified. The concept of selective clonal expansion of initiated cells by tissue specific agents has applicability to all model systems where promotion has been demonstrated. Our mechanistic studies have provided insight for other investigators to pursue tissue specific mechanisms of promotion in other models. Furthermore, the insights obtained from these studies have guided our experiments on the mechanism of action of promotion inhibitors, in particular retinoids, steroids, and protease inhibitors. In vivo studies from this project have defined a discrete, promotion independent step of malignant conversion. This is likely a genetic change and the genes involved are under study. Since tumors become life threatening once malignant conversion occurs, molecular understanding and prevention of this change could have profound effects on cancer mortality.

Proposed Course:

This project represents an integrated, comprehensive approach to understanding the biological changes associated with initiation and promotion of carcinogenesis and their underlying molecular mechanisms. Future studies are a logical extension of each component of the overall approach. In order to understand the regulation of normal epidermal differentiation, the calcium-modulated culture model will continue to be studied in detail. Ionic changes, which appear to be important in differentiation, will be analyzed by additional studies on ion flux and measurements of intracellular pH changes. Since these changes may involve NaK-ATPase, the activity of this enzyme will be studied under various Ca^{++} conditions and after TPA exposure. If antibodies can be obtained, the phosphorylation state of NaK-ATPase will be assessed under conditions of induced differentiation. Since our studies suggest that activation of protein kinase C is common to both Ca^{++}- and TPA-induced differentiation, protein phosphorylation patterns will be analyzed in detail by 2-D gel analysis assisted by computer. These studies will be performed in collaboration with Drs. Snorri Thorgeirsson and Peter Wirth. Direct isolation of protein kinase C from normal and altered epidermal cells, activity measurements and subcellular localization will be studied. New proteins which are synthesized during differentiation will be categorized, and modulated proteins (synthesis or phosphorylation) which change similarly for both inducers (TPA or Ca^{++}) will be studied in depth in the hope of identifying their functions. Further efforts to clarify the role of the calcium-binding proteins or associated antigens in epidermis will be made through studies at the protein level and through sequence of the cloned genes and the study of gene expression at the molecular level. Monoclonal and polyclonal antibodies to the basal cell proteins or synthetic peptides will be raised in rabbits and mice and used to isolate these proteins. The results obtained with normal keratinocytes will be compared to results of similar studies performed on preneoplastic and neoplastic keratinocytes.

Transformation studies utilizing resistance to induced differentiation will be expanded. Additional chemicals of varying initiating activity and ionizing radiation will be tested. Modification of the target cells at the time of carcinogen exposure will be utilized to attempt to enhance or inhibit the transforming event. Modifiers will be chosen which are known to alter initiation in mouse skin _in vivo_. Modifiers which can alter the extent or pattern of carcinogen binding to DNA will also be utilized to determine the effect on initiation. Immunological assays, developed in this laboratory (see project Z01-CP-05177-01-CCTP), will be used to monitor binding. The effects of split doses of UV will be studied in greater detail to determine the mechanism of enhancement of focus production by this treatment protocol. Analysis of cell cycle changes, persistance of sensitivity to additional exposures after a single exposure and DNA repair kinetics will be analyzed and a variety of split dose regimens will be tested. Further attempts to enhance focus formation with tumor promoters will be made.

Mouse skin will be exposed to a variety of treatment regimens _in vivo_, and epidermal cells from treated skin will be isolated and studied _in vitro_. Progression from the initiated cell to the malignant cell will be systematically studied _in vitro_ to elucidate the temporal sequence of this change and to examine the

capability of additional carcinogen or promoter treatments to accelerate progression. The development of clonal transformation assays will continue with the testing of epidermal cell lines with a high cloning efficiency and the use of feeder layers to enhance clonal growth of primary cells.

The mechanism by which ras gene expression and the p21 transforming protein can alter proliferation, differentiation, and tumorigenicity will be explored at the molecular level. Combined treatments with chemicals and viruses will be performed under conditions where each treatment alone is insufficient to transform cells to tumorigenicity. Harvey or Kirsten sarcoma viruses will be used to infect mouse skin followed by treatment with tumor promoters. Parallel studies using labeled DNA probes to known oncogenes will be conducted to analyze for expression of these genes in various states of altered differentiation and transformation. Transfection of DNA from altered cells into normal basal keratinocytes will be performed followed by high Ca^{++} selection in order to isolate genetic information which can impart differentiation resistance to normal keratinocytes. Human tumor DNA will be used to facilitate the isolation of transfected genes. In addition, cloned oncogenes derived from tumors or cloned genes from oncogenic viruses will be tested by transfection methodology using differentiation resistance as a selectable marker.

The Ca^{++} resistance marker selects for a preneoplastic property. Our in vivo studies indicate that at least two genetic changes are required for malignancy. We will attempt to develop an in vitro assay for the conversion (benign to malignant) step using repeat treatments with carcinogens on differentiation-altered foci obtained after a single exposure. Retreated foci will be tested in vivo for tumorigenicity. If successful, an analysis of the genetic changes involved will be performed by transfecting DNA from the tumorigenic cells into non-tumorigenic Ca^{++} resistant cell lines as recipient. Papilloma cell lines will be used in similar studies.

The observation that retinoids induce a unique epidermal transglutaminase which may be causally related to the modulating effect of retinoids on epidermal differentiation and tumor promotion will be pursued at the molecular level. Both the normal transglutaminase and the retinoid-induced enzyme will be purified and antibodies will be produced. Furthermore, using cells with high activity for each enzyme, attempts to clone the two genes involved (assuming two gene products) will be made. If successful, the cDNA probes generated will be used to analyze the level of regulation involved in each induction and ultimately to facilitate characterizaton of the transglutaminase gene family. Probes will be used to characterize changes in expression in transformed cells. Experiments will also be performed to identify and characterize transglutaminase substrates and to examine their modulation in transformation and under the influence of retinoids.

Our studies on phorbol ester effects on epidermal cells will continue to focus on the molecular mechanism of induced differentiation. Future studies already described will examine the effects of promoters on protein synthesis and phosphorylation, phospholipid turnover, and activation and localization of protein kinase C. Changes in lipid environment under different maturation states will be explored to assess the effects on TPA binding to its receptor.

The effect of TPA on ion fluxes and NaK-ATPase will be studied. Reconstruction experiments with normal and initiated cells or normal and papilloma cells will assess the ability of phorbol esters to select initiated cells from a mixed population. Similar studies will be performed with agents such as mezerein and teleocidin. Other classes of promoters will also be analyzed.

In vivo experiments will be designed to extend our observations of the requirements for carcinoma formation. Treatment schedules will be reversed to test for obligate sequences of various stages in cancer development. Dose response and treatment duration experiments will analyze the sensitivity of the conversion step to carcinogen exposure. Inhibitors of malignant conversion will be sought and promoting agents other than phorbol esters will be examined to determine their potential to convert papillomas to carcinomas. These results will be useful for establishing the relevant biology in vivo which can then serve as a guide to conducting mechanistic experiments on a conversion assay established in vitro.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP04798-15 CCTP

PERIOD COVERED
October 1, 1984 to September 30, 1985

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)
Metabolism and Mode of Action of Vitamin A

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	L. M. DeLuca	Research Chemist	LCCTP NCI
Others:	K. Creek	Staff Fellow	LCCTP NCI
	F. Huang	Expert	LCCTP NCI
	D. Rimoldi	Visiting Fellow	LCCTP NCI
	U. Lichti	Expert	LCCTP NCI

COOPERATING UNITS (if any)
National Institute of Environ. Health Sciences, Research Triangle Park, NC (A. M. Jetten); Univ. of Rome, Italy (S. Adamo); Microbiological Associates, Bethesda, MD (E. F. Spangler)

LAB/BRANCH
Laboratory of Cellular Carcinogenesis and Tumor Promotion

SECTION
Differentiation Control Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
7	5	2

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Maintenance of epithelial differentiation is one of the biological functions of vitamin A and explains current interest in this nutrient as a chemopreventative agent of epithelial cancer. Therefore, current research efforts center on the elucidation of possible sites and mechanisms of action of the vitamin. These were pursued at the following levels: 1) Protein glycosylation: under conditions of vitamin A deficiency, the _in vivo_ incorporation of mannose into guanosine diphosphomannose, dolichylphosphatemannose and lipid-linked oligosaccharides was markedly decreased and resulted in the accumulation of shorter oligosaccharides on lipid-linked intermediates involved in protein glycosylation. At the same time an accumulation of mannose and dolichylphosphate was also observed, suggesting that vitamin A regulates protein glycosylation by controlling the level of GDP-mannose and dolichylphosphate mannose in the membrane. During this work a new mannolipid, phosphatidyl-mannose, was found to be synthesized _in vitro_ by liver microsomal membranes. 2) Keratin gene expression: we have employed a tracheal organ culture system and have reproduced the _in vivo_ phenomenon of squamous metaplasia during culturing under vitamin A free conditions as well as after carcinogen treatment. Vitamin A deficient tracheas were shown by immunoblot analysis to contain keratins of 50, 48, 46.5 Kd detected with the antibody AE_1, and 58, 56, and 52 Kd detected with AE_3. These proteins were either absent or present in much less quantity in control tracheas. In deficient tracheas 60 Kd keratin was found to be located specifically in squamous suprabasal cells, and 55 and 50 Kd keratin proteins were found in a basal cell compartment. Following carcinogen exposure, the appearance of 60 Kd keratin and the enhanced expression of 50 and 55 Kd keratins preceded the squamoid metaplastic response as detected morphologically. Both the keratin changes and the morphological changes were prevented by retinoid treatment. 3) Retinoid transport: a new assay to separate the cellular retinol binding protein from the cellular retinoic acid binding protein by HPLC on Mono Q columns was developed.

PHS 6040 (Rev 1/84)

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

Luigi M. De Luca	Research Chemist	LCCTP NCI
Kim Creek	Staff Fellow	LCCTP NCI
Freesia Huang	Expert	LCCTP NCI
Donata Rimoldi	Visiting Fellow	LCCTP NCI
Ulrike Lichti	Expert	LCCTP NCI

Objectives:

Vitamin A and some of its synthetic analogs (retinoids) have been shown to act as chemopreventative agents during the promotion phase in experimentally-induced epithelial cancer in several animal model systems. Therefore, an investigation of their biochemical mode of action may reveal key points in our understanding of the biochemical steps involved in the control of tumor promotion. Such investigation was conducted along the following lines:

1. To determine the mechanism by which retinoids influence the glycosylation of proteins.

2. To study the synthesis of keratin polypeptides during vitamin A deficiency in hamster tracheas *in vivo* and in organ culture.

3. To study carcinogen-induced changes in morphology and keratin synthesis in cultured trachea and their prevention by retinoids.

4. To study retinoid transport and metabolism in epithelial tissues.

Methods Employed:

HPLC Analysis of Mannolipids. This method was originally designed for studying the synthesis *in vitro* of Dol-P-Man and Ret-P-Man from exogenous Dol-P and Ret-P. The chromatographic separations were conducted on an Altex programmable liquid chromatography system; OD_{325} was monitored with a Hitachi variable wavelength spectrophotometer and radioactivity monitored by a Flow-One Model HS radioactivity flow detector. The chromatographic column used in these studies was the anion exchange Mono Q HR 5/5 with a gradient 0 to 50 mM ammonium acetate in 99% methanol. This method effectively separates authentic Dol-P-Man, Ret-P-Man, and Ret-P with respective elution times of approximately 13, 29, and 36 min. Mannose was recovered in the column flow-through while both mannose phosphate and GDP-mannose remain bound to the column.

Separation of Cellular Retinol Binding Protein From Retinoic Acid Binding Protein by High-performance Liquid Chromatography on Mono Q columns. The anion-exchange column (5 cm x 5 mm diameter) Mono Q was equilibrated with 5 mM Tris-HCl, pH 8.0. The column was connected to an Altex Model 110A programmable liquid

chromatographic system. The system is connected in series by a Hitachi variable-wavelength UV monitor and by a radioactivity flow detector. Counting efficiency for tritium on this system is about 30%. The flow rate was constant at 1.5 ml per minute and generated a back pressure of less than 1000 psi. Solvent A was 5 mM Tris-HCl at pH 8.0 and solvent B was 0.3 M NaCl in 5 mM Tris-HCl, pH 8.0. A gradient designed for this purpose permits the elution of CRABP at 12.6 min at 60 mM NaCl and of CRBP at 22.3 min at 130 mM NaCl. Free retinol and retinoic acid remain on the Mono Q column under these conditions and are usually eluted with 100 ml of 99% methanol.

Techniques for the Study of Keratin Gene Expression in Tracheal Organ Culture. These comprised, in addition to established techniques of organ culture in the presence and absence of vitamin A, the use of various mono- and polyclonal antibodies against purified specific keratins from mouse skin or from human skin. These antibodies were used in immunofluorescence and immunoblotting studies.

Major Findings:

A. Background and Rationale of Glycosylation Studies

Vitamin A and its derivatives (collectively termed retinoids) play an essential role in the maintenance of normal cellular differentiation and proliferation. Numerous studies in several model animal systems have shown that retinoids can act as chemopreventive agents during the promotion phase of experimentally induced epithelial cancer. Furthermore, retinoids are important for growth of normal cells and have been shown to modify growth and adhesive properties of transformed cultured cells in the direction of the normal phenotype.

Studies conducted in a variety of tissues indicate that in normal physiology the vitamin may somehow be involved in the glycosylation of specific mannose-containing glycoconjugates and in this way affect biological processes such as differentiation. Livers from vitamin A deficient animals show a decrease in mannose incorporation into glycoproteins while excess vitamin A enhanced incorporation of mannose, but not galactose, into liver glycoconjugates. Specifically, the mannosylation of α_1-macroglobulin and α-2_u-globulin seem to be under the control of vitamin A. We were the first to report that membranes from several tissues contained an enzyme that used a phosphorylated form of vitamin A (retinyl phosphate) as an acceptor of mannose from GDP-mannose thereby forming retinyl phosphate mannose. We reasoned that vitamin A may exert its molecular action by acting as a coenzyme in mannosylation and began an investigation to determine whether this biochemical mechanism of action of vitamin A is correct. Our overall goal is that a determination of the biochemical mode of action of vitamin A may provide us with clues to an understanding of the more general role of the vitamin in cellular differentiation, proliferation, and supppression of carcinogenesis.

1. Effects of vitamin A deficiency on liver glycoconjugate synthesis in vivo.

If the function of vitamin A is to act as a mannosyl carrier (i.e., as Ret-P-Man) for the formation of lipid-linked oligosaccharide intermediates involved in glycoprotein assembly, then vitamin A deficiency should have a marked impact on

this pathway. We also studied the incorporation of [2-^{3}H]mannose into liver glycolipids and glycoproteins 20 min after injection of the label intraperitoneally into vitamin A deficient and control Syrian golden hamsters. Post-nuclear membranes were prepared from the livers, and the lipid-linked oligosaccharide (LLO) fraction was extracted with a chloroform-methanol-water mixture. The extract was then analyzed on a thin-layer chromatography system which separates LLO based on size (the smaller the size, the higher the mobility) and the LLO was visualized by fluorography. A remarkable difference in the LLO pattern is evident with vitamin A deficiency causing an accumulation of LLO homologues of a smaller size than in the control. In order to determine the size of the LLO, which accumulate during vitamin A deficiency, we prepared a series of radioactive high-mannose type oligosaccharide standards ranging in size from one to nine mannoses. The standards could be clearly separated based on size by high performance liquid chromatography (HPLC). The LLO isolated from control and A-deficient animals were treated with mild acid to release the oligosaccharides from their underlying lipid moiety and then sized on the HPLC column. Unlike the LLO from control animals which contain a predominant species with a migration of a size larger than the $(man)_9$ standard, presumably of the structure $(man)_9(glc)_3$, an accumulation of oligosaccharides of shorter size migrating in the area of the $(man)_8$ standard were found in vitamin A deficiency. Similarly, when the oligosaccharide chains released by β-endo-N-acetylglucosaminidase H treatment of membrane associated glycopeptides were analyzed by HPLC, an accumulation of smaller size oligosaccharide products was observed during vitamin A deficiency.

We also studied the effect of vitamin A deficiency on the amount of [2-^{3}H]-mannose incorporated into dolichyl phosphate mannose (Dol-P-Man) and GDP-mannose 20 min after intraperitoneal injection of the label. We found a decrease of more than 90% in the labeling of GDP-mannose and an equivalent decrease in Dol-P-Man when vitamin A deficient animals were compared to normal. Others have shown that conditions such as glucose deprivation and energy depletion can cause the accumulation of LLO of a shorter size. We have also found that starvation causes an accumulation of LLO of a shorter size as well as a decrease in GDP-mannose synthesis. However, in studies conducted in well-nourished hamsters prior to any manifestation of vitamin A deficiency, a specific effect of this condition was found on accumulation of dolichyl phosphate in the membrane.

2. Comparison of the Enzymatic Synthesis of Ret-P-Man and Dol-P-Man from Exogenous Ret-P and Dol-P In Vitro.

Many striking similarities exist between the enzymatic synthesis of Ret-P-Man and the polyisoprenoid derivative Dol-P-Man. Dol-P-Man is believed to sequentially donate the final four mannose residues to the lipid linked oligosaccharide intermediate Dol-P-P$(GlcNAc)_2(man)_5$ to generate the Dol-P-P-$(GlcNAc)_2(man)_9$ structure which, following glycosylation, is transferred "en bloc" to a nascent polypeptide chain. We have conducted several experiments which were designed to compare the biosynthetic process of Ret-P-Man and Dol-P-Man. As summarized in Table I, we found that when these reactions are assayed using exogenous acceptors, they are reversible, inhibited by the antibiotic amphomycin, and require $MnCl_2$ for optimal synthesis. We also found that the Thy-E negative mutant of the lymphoma cell line Thy-1, which had previously been characterized

Km for GDP-mannose than Ret-P-Man synthesis. Topological studies using sealed crude microsomes revealed that Dol-P-Man synthesis was inhibited less (33%) by phospholipase C pretreatment of the membranes than Ret-P-Man (98%) synthesis. However, this effect is difficult to interpret since it was not observed in preparations of highly purified endoplasmic reticulum. The obvious question of interest is whether or not the membranes contain a single mannosyltransferase that can utilize both dolichyl phosphate and retinyl phosphate or whether separate mannosyltransferases exist which are responsible for each activity. While conclusive proof awaits purification of the mannosyltransferase(s), the evidence at present favors the existence of a single transferase being responsible for both activities.

TABLE I

COMPARISON OF THE ENZYMATIC SYNTHESIS OF RET-P-MAN AND DOL-P-MAN FROM EXOGENOUS RET-P AND DOL-P IN INCUBATIONS WITH LIVER POST-NUCLEAR MEMBRANES

Criteria	Ret-P-Man Synthesis	Dol-P-Man Synthesis
Reversibility by GDP	yes	yes
Inhibition by amphomycin	yes	yes
$MnCl_2$ requirement	yes	yes
Synthesis by Thy-E$^-$ membranes	no	no
Apparent K_m for GDP-mannose	18 µM	1.6 µM
Inhibition by phospholipase C treatment	98 %	33%

3. Identification of an endogenous mannolipid as phosphatidylmannose.

When liver microsomal membranes are incubated *in vitro* with GDP-mannose, the transfer of mannose to at least two endogenous lipid acceptors results. In these experiments, the membranes are incubated at 37°C in the presence of GDP-[^{14}C]mannose, the membranes are extracted with methanol, the lipid extract separated by TLC developed in chloroform-methanol-water, and the mannolipids subsequently visualized by fluorography. Predominant bands are present which co-migrate with authentic Dol-P-Man and standard Ret-P-Man. The finding that postnuclear membranes isolated from vitamin A-deficient livers contained only 50% of the levels of this endogenous acceptor lipid when compared to controls suggested that it may contain a retinoid moiety. We therefore purified larger amounts of this endogenous mannolipid and compared its properties to those of authentic Ret-P-Man made *in vitro* from exogenous Ret-P. Not only were the two compounds separated by HPLC on an anion Mono Q column, but they exhibited differences in their chemical properties which clearly demonstrated that the endogenous mannolipid was not identical to authentic Ret-P-Man. The finding that the endogenous mannolipid lost its hydrophobic character following mild base hydrolysis suggested the presence of fatty acyl chains. Furthermore, the acidic nature of the mannolipid and its susceptibility to phosphodiesterase digestion suggest the presence of a phosphate group on the acceptor. These data were consistent with the endogenous acceptor being phosphatidic acid which is present in microsomal membranes in low amounts as a critical intermediate in phospholipid metabolism. When exogenous phosphatidic acids containing different

length fatty acyl chains were incubated with GDP-mannose and microsomal membranes, they clearly acted as acceptors of mannose, while controls containing phospholipids with a head group blocking the phosphate moiety did not. The properties of the mannolipid formed in the experiments with exogenous phosphatidic acid were identical in every respect to those of the endogenous mannolipid. These results have demonstrated that the endogenous acceptor in microsomal membranes is phosphatidic acid and that the product formed is phosphatidyl mannose.

B. Background and Rational of Studies on Tracheal Organ Cultures.

Vitamin A is essential for the normal growth and differentiation of epithelial cells. In tracheobronchial epithelium, normally a mucus secreting tissue, deficiency of vitamin A causes squamous metaplasia. This lesion is similar to that observed *in vivo* in tracheas treated with benzo[a]pyrene and occurs in organ cultures of tracheas obtained from hamsters kept for 4 weeks on a vitamin A-deficient diet and cultured in a vitamin A-depleted, chemically-defined medium. Retinoids added to the medium can prevent the development of the lesions and restore mucociliary differentiation. It was of interest to identify keratin subunits in vitamin A-deficient tracheas as part of our laboratory's effort to understand the mode of action of vitamin A in normal epithelial differentiation and its involvement in anticarcinogenesis.

1. Morphological changes of cultured tracheas in vitamin A-depleted medium.

Tracheas from four week old hamsters kept on a vitamin A-deficient diet were cultured in a chemically-defined medium without vitamin A. Before culturing, these tracheas were morphologically indistinguishable from normal tracheas. However, upon cultivation, alterations in the epithelium occurred within one to three days with focal replacement of mucous cells by squamous cells. As culture progressed into the 8th and 9th day, squamous metaplasia became severe. More than 80% of the luminal area was covered with squamous cells and with a stratified superficial layer resembling stratum corneum of mouse epidermis. Such alterations in epithelial differentiation did not occur if retinyl acetate was added to the culture medium at a concentration of 1×10^{-7}M, regardless of whether the tracheas were from 4 week old normal or vitamin A-deficient hamsters. Studies on the addition of retinyl acetate to 8-9 day-cultured deficient tracheas indicated that the squamoid differentiation could also be reversed to normal mucociliary differentiation within one week. Tracheas from normal hamsters did not develop squamous metaplasia within the same period of culturing in vitamin A-depleted medium.

2. Indirect immunofluorescent staining for keratins in trachea with squamous metaplastic lesions.

The monoclonal antikeratin antibodies AE1 (specific for acidic keratins) and AE3 (specific for basic keratins) were used for indirect immunofluorescent staining. Both the areas of squamous metaplastic lesions and the "stratum corneum" reacted strongly with AE3. Living cell layers were highly reactive with AE1, while the stratum corneum was less reactive with the same antibody. The mucociliary epithelium of normal trachea also exhibited a uniform pattern of reactivity toward AE1 and AE3, whereas the stromal portion of the trachea was not stained significantly with any of these anti-keratin antibodies.

3. Immunoprecipitation and immunoblotting of keratins from cultured tracheas.

Results are summarized in Table 2. In order to follow the kinetics of keratin synthesis during the development of squamous metaplasia, tracheas cultured for various periods of time were labeled with [^{35}S] methionine, the cytoskeletal proteins were extracted from labeled tracheas and analyzed by SDS-polyacrylamide gel electrophoresis after immunoprecipitation with rabbit antimouse keratin antibody which recognizes most keratin species. Though squamoid metaplasia had not yet developed in one-day cultured tracheas, an increased synthesis of keratin polypeptides was found. The major species synthesized showed molecular weights of 58, 55, 48, 46.5 and 45 kd.

These keratins were also demonstrated by the following immunoblotting technique. Keratins in cytoskeletal extracts from cultured tracheas were separated on SDS-PAGE, blotted onto nitrocellulose and analyzed immunologically with monoclonal antibodies AE1 and AE3 and with monospecific antisera against keratins of 67, 59, 60, 55 and 50 kd; AE1 detected keratins of 50, 48, 46.5 and 45 kd; and AE3 recognized keratins of 58 and 56 kd, mainly in 1 week or 2 week-cultured squamoid tracheas. These same keratins appeared in much less quantity in one day-cultured tracheas, although these organs already actively synthesized keratins, as evidenced from the results of immunoprecipitation. Tracheas cultured in the presence of vitamin A showed only faint bands of 45 kd reactive with AE1 and of 58 kd reactive with AE3. Monospecific antisera against 60, 55 and 50 kd keratins were tested on blots of tracheal cytoskeletal preparations and recognized bands at 60, 55 and 50 kd in tracheas from vitamin A-depleted medium. Keratins of 55 and 50 kd appeared in reduced quantity in tracheas cultured in retinyl acetate supplemented medium, and 60 kd keratin was definitely not present in these tracheas. The monospecific anti 67 and anti 59 kd keratin sera did not recognize any protein in trachea.

TABLE 2

SUMMARY OF KERATIN EXPRESSION PATTERNS

Antibody	Keratin MW,(Kd)	Normal[b] trachea in vitro	Deficient trachea in vitro	Normal trachea in vivo	Deficient trachea in vivo	Carcinogen[c] treated trachea in vitro
MS[a]	67	-	-	-	-	ND[d]
MS	60	-	++	-	++	++
MS	59	-	-	-	-	ND
AE_3	58	+	++	-	++	ND
AE_3	56	-	++	+	++	ND
MS	55	+	++	+	++	++
MS	50	+	++	+	++	++
AE_1	50	-	++	+	++	ND
AE_1	48	-	++	+	++	ND
AE_1	46.5	-	++	-	++	ND
AE_1	45	+	++	_	++	ND

[a] Monospecific antibody
[b] Results from immunoblot analysis
[c] Results from immunofluorescent staining
[d] ND, not determined

Therefore, we conclude that development of squamous metaplasia due to vitamin A deficiency in cultured tracheas is accompanied by an overall increase in keratin synthesis as well as by the appearance of keratin species not normally found in mucociliary tracheal epithelium, as summarized in Table 2.

4. Morphology of tracheas from normal and vitamin A-deficient hamsters in vivo.

Tracheal sections from 7 week old normal and vitamin A deficient male hamsters were analyzed. Normal tracheal epithelium comprised mainly columnar shaped mucous and ciliated cells extending from the basal lamina to the tracheal lumen. Basal cells were interspersed between the basilar parts of the columnar cells and did not form a continuous layer covering the basal lamina. Morphological studies of the hamster tracheas at 7 weeks of vitamin A deficiency showed cornifying epidermoid metaplasia. Such epithelium consisted of one or more continuous layers of large round basal cells and several layers of suprabasal squamous cells which eventually gave rise to a "stratum corneum."

5. Immunoblot analysis of in vivo tracheal keratins.

Cytoskeletal protein extracts from tracheas of normal and vitamin A-deficient hamsters were analyzed by immunoblot with various anti-keratin sera. Extracts of deficient tracheas contained relatively more keratin proteins than those of normal trachea when the same amount of cytoskeletal proteins (50 µg) were compared. Antibody AE1 revealed the presence of 50, 48, 46.5 and 45 kd keratins, and AE3 detected mainly 58 and 56 kd keratins in the extract of deficient

tracheas. Monospecific anti-60 kd and anti-55 and anti-50 kd sera also recognized their corresponding keratins in deficient tracheas. All these keratins were either in much reduced quantity or absent in normal tracheas (Table 2). Cytoskeletal proteins were also extracted from back skin epidermis of normal and vitamin A-deficient hamsters and were immunoblot analyzed with anti-keratin antisera. In contrast to tracheas, normal epidermis appeared to contain as much keratin as vitamin A-deficient epidermis, both quantitatively and qualitatively.

6. Localization of 60, 55 and 50 kd keratins in tracheas.

Since 60 kd keratin appeared only in tracheas of deficient hamsters, it was of interest to determine whether its expression was limited to squamous differentiated cells. Sections of both normal and deficient tracheas were stained with anti-60 kd keratin serum by indirect immunofluorescent staining techniques. As expected from results of the immunoblot analysis, control tracheas did not appear to contain 60 kd-keratin. On the other hand, the squamous epithelium of vitamin A-deficient trachea exhibited positive staining only in suprabasal cells. This staining pattern was different from that of mouse epidermis where 60 kd was expressed strongly in the basal cells. Indirect immunofluorescent staining with anti-55 and 50 kd keratin serum indicated that, in contrast to 60 kd keratin, 55 and 50 kd keratins were synthesized only in the basal cells. It became clear that the normal trachea possessed merely a few scattered basal cells that made 55 and 50 kd keratins, whereas a continuous and multilayer arrangement of basal cells was observed in the trachea of vitamin A-deficient tracheas.

In conclusion, the morphological changes and pattern of keratins synthesized in vitamin A deficiency-induced squamous metaplasia of cultured tracheas were very similar to those characteristic of deficient tracheas *in vivo*.

7. Morphological changes in carcinogen-induced squamous metaplasia of cultured trachea.

Organ cultures of tracheas from 4 week old normal hamsters when kept in vitamin A-depleted media for 2 weeks did not develop squamous metaplasia. One day after culturing, exposure to either benzo[a]pyrene or DMBA for 48 h induced squamous metaplasia after 2 weeks. Morphologically the squamous metaplasia induced by carcinogens was indistinguishable from that induced by vitamin A deficiency under light microscopy. However, 2 weeks after carcinogen treatment, the lesion occurred only focally and rarely around the entire tracheal circumference; nucleated or anucleated cornified cells were interspersed with areas of minimal change and stratification. Some metaplastic regions comprised cornified cells that were in the process of sloughing off, while others contained maturing anucleated cornified squames that were about to extrude through the overlying minimally changed mucous cells. Importantly, these lesions were prevented when retinoic acid was added to the medium simultaneously with, or right after, the treatment with carcinogens and if present thereafter until the end of 2 weeks of culturing.

8. Expression of 55 and 50 kd keratin during carcinogen exposure.

Normal treacheas do not have a continuous layer of basal cells that specifically express 55 and 50 kd keratins, not even after cultivation of these tracheas for 2 weeks in vitamin A-depleted media, as evident from the indirect fluorescent staining (not shown). However, as a result of carcinogen treatment, an almost continuous layer of basal cells appeared in areas where only minimal changes of morphology had occurred, and these cells were positively stained for 55 and 50 kd keratin. In areas where stratified cells were evident their underlying basal cells were multilayered and were also positively stained. Therefore, expression of 55 and 50 kd keratin in trachea was limited to basal cells and the expansion of basal cells (increased proliferation) following carcinogen treatment resembled that seen in tracheal cultures in the early stages of vitamin A deficiency.

9. Expression of 60 kd keratin during carcinogen exposure.

Keratin of 60 kd was not expressed in normal trachea, and was only detected in suprabasally located squamous cells in vitamin A-deficient tracheas. Two weeks of cultivation of normal tracheas in vitamin A-depleted media failed to induce the expression of 60 kd keratin. On the other hand suprabasally located squamous cells in carcinogen-treated tracheal cultures showed strong reactivity toward anti-60 kd keratin while the "stratum corneum" was less reactive. Interestingly, at some areas where superficial cells were not yet squamoid, a few scattered suprabasal cells were positively stained by this antiserum. It was likely these cells represented an early stage of carcinogen-induced alterations.

In conclusion, upon carcinogen treatment, the normal tracheas in vitamin A-deficient medium underwent transformations resembling that of cultured tracheas from vitamin A-deficient donors maintained in vitamin A-deficient medium. However tracheas from normal donors did not undergo metaplasia in vitamin A-deficient medium in the absence of carcinogen treatment within the same period.

C. Separation of Cellular Retinol Binding Protein (CRBP) from Cellular Retinoic Acid Binding Protein (CRABP).

A one-step procedure to detect cellular [^{3}H]retinoid binding proteins (CRBP and CRABP) from rat testis cytosolic extract was devised. The procedure is based on anion-exchange high-performance liquid chromatography of the cytosolic fraction on columns of Mono Q, which permits elution of CRABP and CRBP at 12 and 22 min, respectively.

The method was applied to determine equilibrium dissociation constants (K_D) and binding capacity (V_{max}).

The binding capacities of 6.89 pmol/mg for CRBP and 7.1 pmol/mg of cytosolic protein for CRABP are within the orders of magnitude as determined by sucrose density gradient and by high-pressure size-exclusion chromatography.

A comparison between this technique and the sucrose density gradient (SDG) technique was made. Parallel analyses of the same cytosol preparation gives similar results for CRBP by Mono Q (24.75 ng/mg) and SDG analysis (17.32 ng/mg) and for CRABP by Mono Q (18.76 ng/mg) and by SDG analysis (19.14 ng/mg), respectively.

Significance to Biomedical Research and the Program of the Institute:

It is the aim of this project to investigate the mechanism(s) by which vitamin A functions in the body. Since vitamin A and its derivatives, the retinoids, are active as preventive agents of certain epithelial cancers, such investigation may yield useful information on mechanisms whereby normal tissue-specific phenotypic expression is maintained by retinoids.

The aim of Part A of this project is to investigate the mechanism by which vitamin A influences the glycosylation of proteins. It is well established that some glycoproteins mediate such important cellular activities as cell to cell recognition and adhesion, while others display hormonal functions in tissue growth and development, and glycoproteins such as mucins offer epithelioprotective functions. Since a clear effect of vitamin A on the adhesive properties of cells, the secretion of mucins, as well as the maintenance of cellular differentiation and proliferation has been established, it is reasonable to propose that the vitamin somehow influences or regulates glycosylation of glycoprotein expression. An understanding of the exact biochemical mechanism by which vitamin A influences glycoprotein biosynthesis may yield important information on how the vitamin helps maintain normal tissue-specific differentiation. Since cancer is fundamentally a disorder of cellular differentiation and proliferation and retinoids serve as chemopreventative agents in certain cancers, the value of understanding their underlying mechanism of action should prove useful in designing new rational approaches to chemotherapeutic management of cancer as well as cancer prevention.

A second aim (Part B) is to understand mechanisms whereby vitamin A controls keratin gene expression. Exposure of the epithelial surface of the hamster respiratory tract to benzo[a]pyrene in vivo eventually results in the development of squamous cell carcinomas, a type of cancer also common in cigarette smokers in the human population. The early changes caused in the experimental model of hamster respiratory cancer after exposure to benzo[a]pyrene include the squamous metaplastic lesion also observed in Syrian golden hamsters kept on a vitamin A-depleted diet. The tracheal organ culture system was utilized to define the molecular changes in keratin expression during vitamin A deficiency and after exposure to carcinogens. The results obtained indicate this system to be of potential usefulness in studying carcinogenesis and its prevention by retinoids. Of particular interest is the finding that carcinogen exposure in vitro causes morphological alterations similar to the in vivo situation. The oncogenic potential of such squamous metaplastic foci is of interest to us, particularly since they are prevented from occurring by in vitro treatment with retinoids. Therefore, the advantage in using the tracheal system resides in its potential to respond to carcinogens, vitamin A deficiency and retinoid treatment by displaying a range of phenotypes from fully epidermoid to mucociliary, a potential not readily observable in tissues such as the epidermis and the intestinal mucosa, which remain either keratinized or mucus secretory.

Whereas the keratin pattern of normal tracheas in vitro is similar to in vivo, certain peptides are expressed specifically under one condition or the other. Expression changes of keratin genes have been previously noted for epidermis in vivo or in vitro. Of significance, however, for our purposes was the identical pattern of keratin gene expression of deficient tracheas in vivo and in vitro (see Table 2). This finding supports the validity of the organ culture model for studying vitamin A action.

The finding that a 60 kd keratin was only expressed in suprabasal cells of vitamin A deficient and carcinogen-treated tracheas and that the onset of synthesis of this particular keratin preceded the squamoid transformation may be of prime importance, in that the detection of this marker keratin may ensure an early diagnosis of the lesion and facilitate preventive efforts. The tracheal culture system is also ideal for probing the enhanced expression of oncogenes by in situ hybridization technique (when these techniques become sufficently sensitive for oncogene probing) during carcinogenesis and for studying anticarcinogenic mechanism of vitamin A.

A third aim of this project (Part C) is to study the transport of retinol and retinoic acid from the blood to the target tissues and within intracellular compartments. Such studies on transport should, in the long run, explain why certain lesions, such as squamous metaplasia of the bronchial mucosa due to cigarette smoking persist in individuals who have a normal vitamin A intake. The possibility that such persistence is due to defective retinoid transport that may cause localized deficiency of vitamin A is an intriguing thought. The assay that we have developed in this past year to separate CRBP from CRABP is one important facet of this problem and will greatly aid further investigations.

Proposed Course:

The glycosylation work (Part A) will be pursued as follows. We will use our newly developed HPLC procedure on columns of Mono Q which separates Ret-P-Man from other mannolipids and from Ret-P to determine whether Ret-P-Man is synthesized in vivo and can be isolated and characterized from hamster liver. Fast atom bombardment mass spectrometry, used to confirm the structure of the in vitro made Ret-P-Man, will be applied to the characterization of the putative physiological compound. An important consideration is that retinoic acid is as active as retinol to maintain normal glycosylation under physiological conditions; however, this compound cannot be reduced to retinol. Therefore, if vitamin A participates as a carrier in mannosyl transfer, the following possibilities may exist: (1) A metabolite of retinoic acid, different from retinol but containing a carbinolic group, may function as well as retinol in the postulated carrier mechanism. (2) Both retinol and retinoic acid may be metabolized to a common compound which then functions as a glycosyl carrier. These possibilities will be probed experimentally by HPLC analysis after in vivo injection of radioactively labeled retinol, retinoic acid and mannose. If a labeled compound having properties of Ret-P-Man can be isolated, we will proceed to characterization by mass spectrometry in the fast atom bombardment mode.

The alternative possibility that control of glycosylation by retinoids is exerted at some other level will be examined if we fail to isolate and characterize glycosyl retinoid phosphate synthesized under physiological

conditions. Our strategy in this latter approach would be to concentrate our efforts in defining the various steps by which retinoid treatment eventually results in altered glycoprotein synthesis and secretion, thereby influencing cell adhesion and differentiation phenomena.

The work on tracheal organ culture (Part B of this project) will be pursued as follows. This project has confirmed the antagonistic actions of retinoids and carcinogens at the morphological level in tracheal organ cultures, and has permitted the identification of a specific keratin species (60 kd) which is expressed early after carcinogen treatments. We will determine whether other keratin species synthesized in carcinogen-treated tracheas are similar to or different from those detected in vitamin A-deficient tracheas. These analyses will initially be performed by immunoblot transfer techniques using specific anti-keratin antibodies. Moreover, our ability to detect a population of suprabasal cells which appear to synthesize 60 kd keratin immediately after carcinogen treatment has stimulated our interest in probing the expression of the 60 kd keratin gene, using *in situ* hybridization techniques in collaboration with Dr. Dennis Roop, IVP. A similar approach appears feasible to study the expression of oncogenes. Recent work has suggested an inhibitory effect of RA on c-*myc* in embryonal carcinoma cells and in the human leukemia cell line HL-60 concomitant with differentiation responses due to the retinoid. Moreover, expression of c-*myc* and other oncogenes appears enhanced in some lung cancer tissue. Therefore, we plan to probe oncogene expression in our *in vitro* tracheal organ culture system. The technique of *in situ* hybridization will be employed with probes corresponding to ras^{Ha}, ras^{Ki}, *myc*, *myb*, *fes*, *fos* and *abl* oncogenes. Our purpose is to establish the timing of the enhanced expression of the oncogenes (if enhanced) in relation to the time course of the hyperplastic and squamous metaplastic response to carcinogen and/or Vitamin A deficiency. The effect of the tumor promoter, TPA, on potentiating the squamoid transformation, on activating keratin genes and enhancing oncogene expression (if any) in this organ culture system will also be studied. It will be of interest to study whether or not the TPA effect requires pre-exposure of the organ to an initiator to elicit expression of specific keratin gene products such as the 60 kd protein and/or oncogenes. A detailed time-course of the effect of retinoid treatment on the reversal of squamous metaplasia and suppression of keratin and oncogene expression (if any) will also be carried out.

Finally, tumorigenicity of the carcinogen-treated tracheal epithelium in nude mice will be tested to assess whether exposure to carcinogen sufficient to produce squamous metaplasia is also sufficient to cause tumor formation *in vivo*. It is expected that multiple exposure will be required.

The third aim (Part C) of this project will be pursued as follows. Little information exists as to the mechanism by which retinol and/or retinoic acid is delivered from serum retinol binding protein (RBP) and enters in vitamin A responsive cells. Furthermore, once it enters the cell the intracellular pathway the vitamin follows, or even the exact subcellular compartment(s) in which it exerts its action are unknown. Primary mouse epidermal cells exhibit an induction of transglutaminase activity and an inhibition of cornified envelope formation following exposure to retinoic acid. Since the responsiveness of epidermal cells to retinoic acid is well documented, this cell system should prove useful for the following proposed course of study. RBP will be purified

from serum using immobilized antibody to RBP. Radioiodinated RBP will be used to determine whether primary mouse epidermal cells contain a specific and saturable cell surface receptor for RBP recognition. A cooperative influence of binding of RBP to the putative receptor following formation of retinol RBP complex will be explored. If RBP receptor can be definitively demonstrated, purification of the receptor, following detergent extraction from epidermal cell membranes, will be attempted by affinity chromatography on immobilized RBP in order to raise rabbit antibodies to the receptor. Additionally, the route of delivery of retinol and retinoic acid into the cell will be followed using ^{3}H-labeled retinoid bound to RBP. In particular, we wish to determine whether RBP enters the cell with ligand bound and follows the well-characterized route of receptor mediated endocytosis as has been determined for a variety of ligands such as EGF, transferrin, insulin, and low density lipoprotein. An alternative possibility is that the vitamin enters the cell directly by insertion through the plasma membrane and that the RBP bound at the cell surface never enters the cell. To distinguish between these possibilities the [^{3}H]retinol-[^{125}I]RBP complex will be bound to cells at 4°, excess ligand removed by washing, and the cells warmed to 37° to allow internalization and delivery of the vitamin to the cytoplasm. By washing the cells with excess cold RBP or by stripping the cell surface by mild trypsinization, it should be possible to determine whether the [^{125}I]RBP actually enters the cells. Whatever the mechanism of entry into the cell, the next step will be to follow the intracellular fate of the [^{3}H]labeled vitamin. Using our recently developed HPLC technique for identification of cellular retinol binding protein (CRBP) and cellular retinoic acid binding protein (CRABP), we will determine whether the ligand becomes bound to these proteins following delivery to the cell interior. Finally, using standard subcellular fractionation techniques such as sucrose density centrifugation as well as fractionation on isosmotic self-forming gradients of Percoll we will do a time course study to follow the intracellular traffic route followed by the vitamin once it reaches the cell interior. Identity as well as purity of subcellular fractions will be monitored using appropriate enzymatic marker assays as well as morphometric analysis of electron micrographs of the various subcellular fractions. The role of CRBP and CRABP in retinol and retinoic acid intracellular transport will be studied. An understanding of the mechanism of vitamin A entry into epidermal cells as well as its intracellular destiny may yield valuable clues as to whether the vitamin regulates the expression of genetic information in these cells or whether it acts at an epigenetic level.

Publications

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05177-04 CCTP

PERIOD COVERED
October 1, 1984 to September 30, 1985

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)
Use of Immunological Techniques to Study Interaction of Carcinogens with DNA

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	M. C. Poirier	Research Chemist	LCCTP	NCI
Others:	S. H. Yuspa	Chief	LCCTP	NCI
	H. Huitfeldt	Visiting Fellow	LCCTP	NCI
	E. Reed	Special Assistant for Science	DCT	NCI
	C. Litterst	Research Chemist	LMCP	NCI
	R. Ozols	Chief	MB	NCI
	T. Hamilton	Clinical Associate	MB	NCI

COOPERATING UNITS (if any)
MIT, Boston, MA (S. Lippard); Univ. of Texas Medical School, Houston, TX (J. M. Hunt); Univ. of North Carolina, Chapel Hill, NC (D. Kaufman and R. Paules); NCTR, Jefferson, AR (F. A. Beland and J. Young)

LAB/BRANCH
Laboratory of Cellular Carcinogenesis and Tumor Promotion

SECTION
In Vitro Pathogenesis Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
4.5	3.0	1.5

CHECK APPROPRIATE BOX(ES)
☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)
Antibodies specific for carcinogen-DNA adducts have probed the nature, extent, and consequences of in vitro and in vivo DNA modification. DNAs substituted with 2-acetylaminofluorene (AAF), benzo[a]pyrene (BP), or cis-diamiminedichloroplatinum II (cis-DDP) were analyzed by quantitative immunoassays able to detect one adduct in one hundred million nucleotides, and by immunohistochemical procedures developed to localize adducts in situ. In hepatic DNA of rats fed a carcinogenic dose of AAF for 4 weeks, adduct accumulation reached a plateau at 2-3 weeks and adducts were shown by immunohistochemistry to be primarily localized in the periportal areas. During 4 subsequent weeks on control diet, adduct removal was biphasic. A computer-derived pharmacokinetic model consistent with this data proposed that adducts are formed into two genomic compartments, one from which adducts are removed rapidly and another from which they are removed slowly. In contrast to the high levels of AAF adducts formed in rat liver DNA, at least 50-fold lower adduct quantities were formed in the DNA of mouse epidermis and cultured mouse epidermal cells exposed to initiating doses of BP. When activated forms of both carcinogens were utilized in the keratinocyte focus assay N-acetoxy-AAF yielded more adducts per molar concentration than the BP derivative but no differentiation-altered foci formed in N-acetoxy-AAF treated cultures. Nucleated peripheral blood cell DNA was obtained from cancer patients at multiple times during courses of cis-DDP therapy, and a total of 223 samples were analyzed. Of these, 23 untreated control samples were negative, and 46% of the 200 samples from patients receiving cis-DDP were positive. Adduct accumulation, in positive patients, occurred as a function of total cumulative dose, suggesting relatively slow adduct removal. Disease response data on 47 patients indicated that individuals with adduct levels greater than 200 attomoles/μg DNA have a very high (65%) rate of complete response to therapy. Parallel experiments in animal models have demonstrated that the same adduct forms in kidney, gonads, and tumors of rats and mice in direct relation to dose.

PHS 6040 (Rev. 1/84)

PROJECT DESCRIPTION

Names, Titles, Laboratory, and Institute Affiliations of Professional Personnel Engaged on this Project:

M. C. Poirier	Research Chemist	LCCTP	NCI
S. H. Yuspa	Chief	LCCTP	NCI
E. Reed	Special Assistant for Science	DCT	NCI
H. Huitfeldt	Visiting Fellow	LCCTP	NCI

Objectives:

To develop specific and sensitive quantitative and morphological immunoassays for the investigation of carcinogen-DNA interactions. Studies are directed toward quantitative and qualitative analyses of covalent DNA adduct formation and removal, and localization of adducts at the cellular and subcellular levels. These data are correlated with biological consequences of chemical carcinogen exposure, including cell transformation and tumorigenesis. In the case of the chemotherapeutic agent cis-diammine-dichloroplatinum (II) (cis-DDP), biological end points include chemotherapeutic efficacy and short- and long-term toxicity.

Methods Employed:

Both in vivo carcinogen exposure of experimental animals and carcinogen treatment of cultured cells are employed to pursue the objectives. Tissues and cells obtained from individuals environmentally exposed to carcinogens or from patients given cancer chemotherapeutic agents are also utilized. The chemical synthesis of radiolabeled and unlabeled DNA-carcinogen adducts and their purification by column chromatography are currently performed. Isolation of macromolecules for carcinogen binding and repair studies utilize density gradient centrifugation. Antibodies are produced by injection of purified antigens into rabbits. A variety of immunological techniques are employed, including the qualitative procedures of immunofluorescence and immunochemical electron microscopy and the quantitative radioimmunoassay (RIA) and enzyme-linked immunosorbent assay (ELISA).

Major Findings:

Interactions of various carcinogens with DNA have been studied in cultured cells, animal organs, and human tissues by a unique immunotechnology pioneered in this section. Rabbit antibodies have been elicited against protein-conjugated carcinogen-nucleoside adducts or methylated-BSA-complexed modified DNAs. The high-affinity antisera obtained have been used to develop radioimmunoassays (RIA) and enzyme-linked immunosorbent assays (ELISA) able to detect as little as one adduct in 10^8 nucleotides. Morphological procedures such as immunofluorescence and immunoelectron microscopy have the potential to demonstrate carcinogen-DNA adducts localized within subpopulations of cells in a complex tissue or within unique sites of the DNA molecule. The three areas of ongoing intensive investigation in this laboratory are as follows: 1) elucidation of mechanisms by which the major rat liver DNA adducts are formed and removed during chronic feeding of a carcinogenic 2-acetylaminofluorene (AAF) regimen; (2) comparison of DNA adduct

formation and removal profiles in mouse epidermis subsequent to topical application of initiating BP doses, and in cultured mouse keratinocytes which form differentiation-altered foci in response to benzo[a]pyrene (BP) or BP-anti-diol epoxide (BPDE I) exposure; and (3) investigation of cis-diamminedichloroplatinum (II) (cis-DDP)-DNA adducts in the nucleated blood cells of testicular and ovarian cancer patients receiving cis-DDP chemotherapy, and in tumor bearing animal models.

Rabbit antisera specific for guanosin-(8-yl)-2-acetylaminofluorene (G-8-AAF) and guanosin-(8-yl)-2-aminofluorene (G-8-AF) have been utilized in competitive RIAs with [^{3}H]G-8-AAF and [^{3}H]G-8-AF tracers to assay for liver DNA adducts during chronic AAF feeding. By feeding nonradioactive and radioactive AAF for varying periods of time it has been possible to compare adduct accumulation both at the beginning and the end of a 4-week period of carcinogen administration. In addition, adduct removal and persistence could be monitored during a subsequent 4 weeks of feeding control diet. These studies have previously been performed in collaboration with Drs. B. Laishes and J. Hunt and have recently culminated in a pharmacokinetic analysis generated by analog-digital hybrid computer in collaboration with Drs. J. Young and F. Beland of the National Center for Toxicological Research. The model postulates zero order kinetic adduct formation and first order kinetic adduct removal from two compartments, one susceptible to fast C-8 adduct removal (K = 0.213 day^{-1}) and another from which these adducts are removed much more slowly (K = 0.026 day^{-1}). The total number of adducts formed in the fast removal compartment during one month of AAF administration (1070 fmol/µg) was five times greater than in the slow compartment (232 fmol/µg), and the persistent adducts observed at 56 days (85 fmol/µg) were localized in the latter region. Overall, the removal of adducts formed during continuous AAF feeding was very efficient since >93% of all the adducts formed during 28 days of AAF exposure were removed by the end of a subsequent month on control diet. At the present time, the nature of the two differentially-removing compartments remains obscure, although likely possibilities include different cell populations, specific areas within the liver lobule and different regions within the DNA. Further investigations to explore a number of these possibilities are in progress.

Frozen sections of livers from animals fed AAF or control diet have been analyzed by immunofluorescence and immunoperoxidase techniques. Nuclear and perinuclear staining of hepatocytes are seen after feeding AAF for 3 or 28 days. This staining is specific for the presence of C-8 adduct and is not found in control liver sections or in liver sections of AAF-fed animals reacted with G-8-AF-absorbed anti-G-8-AF. Specific staining was not detected when the concentration of adduct (determined by RIA) dropped below 30 fmol/µg DNA or about 10^5 adducts per cell. The antiserum used for these studies is specific for G-8-AF, which is the predominating hepatic C-8 adduct at 3 (80%) and 28 days (100%) of AAF feeding. The overall pattern of adduct distribution in livers of AAF-fed male rats was distinctly non-uniform, but similar, in both frozen sections and ethanol-fixed paraffin-embedded sections stained by either immunofluorescence or immunoperoxidase. A predominance of nuclear staining, indicating the highest adduct concentrations, was demonstrated in the cells of the periportal areas (Zone 1). In contrast, staining was virtually nonexistent in the centrilobular areas, indicating much lower adduct concentrations in Zone 3. In addition, adduct concentrations were not detectable in altered foci positive for γ-glutamyltranspeptidase in livers of rats fed AAF for eight weeks.

An antiserum elicited against DNA substituted with only the trans-(7R)-N^2-(10-[7β, 8α, 9α-trihydroxy-7,8,9,10-tetrahydrobenzo[a]pyrene]-yl)-deoxyguanosine (BPdG) adduct has a higher affinity for BPdG-modified DNA than for the individual adduct. The ability of this antiserum to recognize adducts in nonhydrolyzed DNA makes it particularly useful for morphological studies. In collaboration with R. S. Paules and D. G. Kaufman, electron microscopic (EM) visualization of BPdG adducts on *in vitro*-modified calf thymus DNA has been achieved using Fab fragments of specific antiserum and ferritin-conjugated goat-anti-rabbit IgG. Quantitative detection was obtained by EM measurement of approximately 1500 DNA fragments per sample, since comparison by both EM and immunoassay yielded similar results. In addition, quantities of adducts on DNA from C3H 10T1/2 cells exposed to (+)7β, 8α-dihydroxy-9β,10β-epoxy-7,8,9,10-tetrahydrobenzo[a]pyrene (BPDE I, anti-isomer) were determined by ELISA and EM to be virtually identical. Thus, the EM immunotechnology will be feasible for use with DNA samples generated by *in vivo* exposure.

The development of fluorescent ELISA methods has enhanced the detectability of BPdG to one adduct in 10^8 nucleotides. This has been achieved through the use of methylumbelliferyl phosphate, and a fluorescence-detecting microtiter plate reader. Studies using this assay have been designed to compare mechanisms of BPdG adduct formation and removal in mouse epidermis *in vivo* and in cultured mouse epidermal cells. Adduct formation was dose-dependent in both systems, although a binding plateau was achieved *in vivo*, while higher doses caused linear adduct formation and cell death *in vitro*. A remarkable feature of both *in vivo* and *in vitro* experiments is that similar levels of BPdG adducts, 1-8 fmol adduct/μg DNA, were found in both mouse epidermis after topical application of initiating doses of BP and cultured keratinocytes at doses which induce the formation of differentiation-altered foci. The kinetics of BPdG removal were also similar ($T_{1/2}$ = 24-48 hr) in both epidermis and the cultured keratinocytes. Experiments in which DNA turnover was monitored by ^{14}C-thymidine prelabeling suggested that in the epidermal cultures, adduct removal by differentiating cells was more rapid than removal in basal cells. Both *in vivo* and *in vitro* the quantity of BPdG remaining on the DNA after the initial removal and during the period of oncogenic expression, is only about 400 adducts per cell. Thus, striking similarities exist between the processing of genomic damage *in vivo* during BP-induced initiation and the production of BP-induced differentiation-altered foci *in vitro*.

Since our *in vivo* data have shown that tumorigenesis is associated with formation and removal of many more adducts in liver than in skin, we have studied the relative potencies of AAF and BP using the keratinocyte differentiation-altered focus assay and activated forms of the two carcinogens. These experiments have shown that a small number of BPdG adducts are associated with a large number of BPDE I-induced foci at nontoxic doses. In contrast, the activated derivative of AAF, N-acetoxy-AAF, induced virtually no foci but gave equal or greater numbers of adducts. Additional studies indicated that removal rates were similar for both agents and that differential cytotoxic effects would not explain the greater potency of BPDE I in this system. Since the hydrocarbons are considerably more effective skin initiators than the aromatic amines, this *in vitro* model provides a unique opportunity to explore carcinogen specificity.

cis-Diamminedichloroplatinum (II) (cis-DDP) is a remarkably potent chemotherapeutic agent which induces the formation of an intrastrand N^7-deoxy(GpG)-diammineplatinum adduct as a major fraction of total platinum bound to DNA. In collaboration with S. Lippard, we have elicited a polyclonal antibody specific for this adduct and developed an ELISA capable of detecting 25 attomoles of adduct/μg DNA. Because the antibody is specific for DNAs modified with certain chemotherapeutically-effective cis-reacting analogs of cis-DDP, structurally similar bidentate intrastrand adducts are believed to play a role in the tumoricidal efficacy of these compounds. Using the anti-cis-DDP-DNA-ELISA, we have analyzed 223 samples of DNA extracted from nucleated peripheral blood cells (buffy coat) of controls, and testicular and ovarian cancer patients at multiple times during cis-DDP treatment. Of these, all 23 samples from untreated controls were never positive, and 46% of the 200 samples from cis-DDP-treated patients were positive. Patients on their first course of chemotherapy receiving cis-DDP on 21- or 28-day cycles (5 days of drug infusion followed by 2 or 3 drug-free weeks) accumulated DNA adducts as a function of dose and increasing cycle number. Thus, the removal time for measurable adducts formed during one cycle may be more than 28 days. An analysis of disease response data for 47 patients showed that individuals forming high levels of cis-DDP adducts ($\geq$200 attom/μg DNA) were more likely to undergo complete remission than those forming fewer adducts or no adducts at all.

Selected tissues of rats and mice were examined following intraperitoneal injections (IP) of cis-DDP in collaboration with C. L. Litterst. Tissues known to be targets for cis-DDP therapy or toxicity, such as kidney, gonad, and tumor, were chosen. Early studies demonstrated that interindividual variability was substantially diminished when animals were fasted overnight rather than fed ad libitum. Results using fasted animals have shown that adduct levels were highest in kidney, somewhat lower in tumor (subcutaneous walker sarcoma) and lowest in gonads, when measured 4h after a single IP injection of cis-DDP. When males were injected 3 times at weekly intervals, adducts accumulated in the testes with multiple exposures but decreased concomitantly in kidneys of the same animals. In addition, males formed more adducts in kidney and gonadal tissues than females. Other studies are being performed in collaboration with R. F. Ozols and T. C. Hamilton. In nude mice bearing an intraperitoneal human ovarian carcinoma implant, cis-DDP-DNA adducts were monitored in kidney, solid tumor and ascites 4h following an IP drug injection. All three tissues had equivalent numbers of adducts.

Significance to Biomedical Research and the Program of the Institute:

The development of immunological procedures for the investigation of carcinogen-DNA interactions has provided a powerful tool for the study of this phenomenon. Experimentally, antibodies are more specific and sensitive, and less costly than the conventional radiolabeled probes used for such studies. Standard carcinogenesis protocols need not be modified for quantitation of DNA-binding since prolonged sequential administration can be monitored. Morphological approaches can be employed to determine inter- and intracellular distribution of adducts. Intensive investigations of specific experimental models of tumor induction, such as AAF-induced tumors in rat liver or BP-induced initiation in mouse epidermis, have provided an opportunity to evaluate cell- or tissue-specific and carcinogen-specific aspects of carcinogen-DNA interactions. The studies designed to monitor for biologically-effective dosages by quantitating cis-DDP-DNA adducts constitute

the first measurement of DNA adducts induced by a chemotherapeutic agent in cancer patients. Since second malignancy is not an uncommon consequence of cancer chemotherapy, and since the survival among testicular patients is high, information generated in this study may form an important data base for evaluating the relationship between DNA damage and human cancer etiology.

Proposed Course:

Future studies will continue to focus on carcinogen-DNA adduct quantitative analysis and immunolocalization in model systems. Ongoing quantitative experiments with liver from AAF-fed rats have been designed to elucidate the nature of the fast and slow removing regions. To this end, elutriation will be utilized to separate different cell types within the liver. Differentiation of genomic regions within liver DNA will be on the basis of repetitive and unique DNA sequences, and eu- and heterochromatin, and will be achieved by gradient centrifugation as well as appropriate enzymatic restriction. Morphological and immunohistochemical studies continuing the investigation of AAF-DNA localization in rat liver will be expanded to include a comparison of preneoplastic liver identified by phenotypic markers and surrounding morphologically-normal liver tissue. A new AAF-DNA antibody now being elicited in guinea pigs should be useful for localizing acetylated C-8 adducts not detected by the antiserum currently in use.

A series of experiments has been designed to determine quantities of carcinogen present on defined sequences of the genome through antibody-detection of adducts. Preliminary data suggest that slot-blotted BP-modified DNA can be detected at a sensitivity of about 25 fmol/μg DNA. As envisioned, future studies will involve separation of specific sequences from total genomic DNA by cDNA hybridizations, and subsequent detection of adducts on these sequences by ELISA. Ideally these experiments would be performed with BPDE I-exposed keratinocytes, and adducts would be determined on genes controlling differentiation under conditions of BP exposure known to induce differentiation-altered foci. Known proto-oncogenes will be studied in the same fashion. Initially, it may be necessary to determine adducts in genes with multiple cellular copies, such as satellite sequences, or to artificially amplify certain sequences by transfection or other selection methods.

The differentiation-altered transformation assay will also be employed to continue the investigation of tissue-related carcinogenesis in keratinocytes. A number of agents which are known to modulate both BP- and AAF-DNA adduct formation will be utilized in an attempt to uncover underlying mechanisms. Furthermore, analogs known to modify the C-8 or N2 positions will be tested to investigate site specificity on epidermal DNA.

A continuation of the human studies will focus on the relationship between cis-DDP-DNA adduct formation and disease response, in anticipation that more effective drug administration may allow the clinician to avoid excessive toxicity. In addition, the kinetics of post-treatment adduct persistence or removal will be followed in a small subset of patients. A collaborative effort with R. P. Perera will simultaneously compare adduct levels with SCEs in post-treatment blood samples from head and neck and lung cancer patients. In collaboration with D. S. Alberts, we will be monitoring buffy coat DNA adducts from individuals

receiving maximally-tolerated dosages of cis-DDP, CHIP, and carboplatinum. The animal studies will attempt to model drug exposure to enhance tumor response and will be directed toward complementing the human studies in a number of areas. The effects of dose schedule and hormonal status on adduct formation and persistence in clinically-significant tissues will continue to be investigated. Two agents which may play a role in the modulation of adduct formation, glutathione and metallothionein, will be studied in cell culture and intact animals.

Publications:

Nakayama, J., Yuspa, S. H. and Poirier, M. C.: Comparison of benzo[a]pyrene-DNA adduct formation and removal in mouse epidermis in vivo and mouse keratinocytes in vitro and the relationship of DNA binding to initiation of skin carcinogenesis. Cancer Res. 44: 4087-4095, 1984.

Paules, R. S., Poirier, M. C., Mass, M. J., Yuspa, S. H. and Kaufman, D. G.: Quantitation by electron microscopy of the binding of highly-specific antibodies to benzo[a]pyrene-DNA adducts. Carcinogenesis 6: 193-198, 1985.

Poirier, M. C.: Review: The use of carcinogen-DNA adduct antisera for quantitation and localization of genomic damage in animal models and the human population. Environ. Mutagen. 6: 879-887, 1984.

Poirier, M.C., Hunt, J. M., True, B. A. and Laishes, B. A., Young, J. F., Beland, F. A.: DNA adduct formation, removal, and persistence in rat liver during one month of feeding 2-acetylaminofluorene. Carcinogenesis 5: 1591-1596, 1984.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05178-04 CCTP

PERIOD COVERED
October 1, 1984, to September 30, 1985

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)
Cellular and Tissue Determinants of Susceptibility to Chemical Carcinogenesis

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	J. E. Strickland	Research Chemist	LCCTP	NCI
Others:	S. H. Yuspa	Chief	LCCTP	NCI
	H. Hennings	Research Chemist	LCCTP	NCI
	D. Roop	Expert	LCCTP	NCI
	J. Harper	Staff Fellow	LCCTP	NCI

COOPERATING UNITS (if any)
Microbiological Associates, Bethesda, Maryland (E. F. Spangler)

LAB/BRANCH
Laboratory of Cellular Carcinogenesis and Tumor Promotion

SECTION
In Vitro Pathogenesis Section

INSTITUTE AND LOCATION
NIH, NCI, Bethesda, Maryland 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.4	2.4	

CHECK APPROPRIATE BOX(ES)
☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)
In vivo carcinogenesis studies have shown that the SENCAR mouse is unusually susceptible to skin carcinogenesis by initiation and promotion. This sensitivity is not a result of differences in carcinogen metabolism between SENCAR and more resistant mouse strains; and grafting experiments show that the enhanced sensitivity is a property of the skin itself, suggesting the usefulness of _in vitro_ studies to elucidate the mechanism. Such studies have shown that cultured epidermal cells of SENCAR and the more resistant Balb/c strain are remarkably similar in a variety of respects, including DNA repair, binding and metabolism of epidermal growth factor, immunological properties, and growth kinetics in response to modulation of carcinogenesis. Recent results of _in vitro_ experiments with foci of cells resistant to Ca^{2+}-induced terminal differentiation following carcinogen treatment suggest that the number of initiated cells induced in SENCAR and Balb/c are equal but that there are qualitative differences in the nature of the initiated cells. Evidence points to enhanced sensitivity to promotion as an important aspect of SENCAR susceptibility. _In vivo_ carcinogenesis studies demonstrate a different papilloma response between SENCAR and CD-1 mice. In CD-1 mice, papilloma incidence continues to rise with continued TPA treatment while in SENCAR, papilloma regression occurs whether or not TPA is continued. Both _in vivo_ and _in vitro_ data support the existence of a constitutively initiated cell population in SENCAR mouse skin but not skin of more resistant strains. The contribution of such cells to SENCAR susceptibility has not been defined.

PHS 6040 (Rev. 1/84)

PROJECT DESCRIPTION

Names, Titles, Laboratory, and Institute Affiliations of Professional Personnel Engaged on this Project:

James E. Strickland	Research Chemist	LCCTP	NCI
Stuart H. Yuspa	Chief	LCCTP	NCI
Henry Hennings	Research Chemist	LCCTP	NCI
Dennis Roop	Expert	LCCTP	NCI
John Harper	Staff Fellow	LCCTP	NCI

Objectives:

To elucidate the cellular and molecular mechanisms of enhanced sensitivity to carcinogenesis in genetically-derived susceptible mouse strains.

Methods Employed:

The SENCAR mouse was developed by a selective breeding protocol for enhanced susceptibility to skin tumors produced by initiation-promotion protocols. In order to elucidate the basis for this susceptibility, skin of SENCAR mice or SENCAR epidermal cells in culture are exposed to carcinogens and tumor promoters. Comparisons are made with BALB/c as a representative resistant strain. For culture of adult mouse epidermal cells, the epidermis can easily be separated from the dermis after flotation of the skin, dermis side down, on a solution of 1 g trypsin per 100 ml phosphate-buffered saline (without calcium and magnesium) for 1 hr at 37°. Epidermal cells are plated on dishes coated with fibronectin and collagen and are cultured in low-calcium medium conditioned by dermal fibroblasts. After treatment with initiating doses of carcinogens, selection for cells resistant to calcium-induced terminal differentiation can be made. Receptor binding studies with cells in culture have used radioactively-labeled epidermal growth factor. Effects of various agents on epidermal cell growth kinetics have been determined in culture by cell counts and thymidine incorporation. The presence of keratins was determined by fluorescent antibody staining and by polyacrylamide gel electrophoresis of radioactively-labeled cell proteins precipitated by anti-keratin antibodies. Gamma glutamyl transpeptidase was determined by histochemical staining. Transglutaminase and ornithine decarboxylase were measured in cell lysates by standard enzyme assays using radioactively-labeled substrates. Cells are tested for tumorigenicity by subcutaneous implantation into nude mice. Initiated and papilloma cells are transplanted to nude mice as reconstituted skin grafts to test behavior and response to tumor promotion *in vivo*.

Major Findings:

The SENCAR mouse was developed by a selective breeding protocol for increased sensitivity to skin carcinogenesis by initiation and promotion. Subsequent studies have shown that the enhanced sensitivity exists for initiators with a

variety of chemical structures, including some not requiring metabolic activation, and does not result from differences in metabolism of polycyclic aromatic hydrocarbons. Indeed, the SENCAR mouse is more sensitive to skin carcinogenesis by a single dose of ultraviolet (UV) radiation than the more resistant CD-1 strain. Skin graft studies demonstrate that the sensitivity of the SENCAR mouse is a property of the skin itself, rather than a systemically mediated phenomenon. This finding validates the use of _in vitro_ studies with cultured skin cells to elucidate the mechanism of SENCAR sensitivity. Such studies have indicated that cultured cells of SENCAR and the resistant Balb/c strain are remarkably similar in a variety of respects. Epidermal cells from these mice do not differ in their ability to repair DNA damaged by UV radiation. SENCAR epidermal cells are comparable to Balb/c to the extent to which they bind epidermal growth factor (EGF) and respond to modulators of EGF binding such as the tumor promoter 12-O-tetradecanoylphorbol-13-acetate (TPA), retinoic acid, and Ca^{2+}-induced terminal differentiation. Langerhans cells, bone-marrow-derived cells normally present in the epidermis, are present in similar numbers in SENCAR and Balb/c and are functionally indistinguishable. In growth kinetics experiments, epidermal cells of SENCAR and Balb/c responded similarly to TPA, retinoic acid, EGF, hydrocortisone, and fluocinolone acetonide at doses commonly used in carcinogenesis experiments. Expression of a number of oncogenes has been found not to differ significantly in SENCAR and Balb/c epidermis under a variety of conditions.

A major achievement in defining the mechanism of SENCAR sensitivity to skin carcinogenesis would be the determination of whether initiation, promotion, or both are involved. Promotion is clearly an important factor. The latent periods before the first papillomas and carcinomas appear in initiation-promotion studies is shorter, often by many weeks, in SENCAR than in resistant mouse strains. SENCAR skin is unusually sensitive to toxic effects of TPA. In _in vivo_ studies, SENCAR, but not Balb/c, mice developed ulcerative skin lesions in response to TPA, necessitating reduction of treatments from twice to once per week. _In vivo_ carcinogenesis studies from several laboratories have shown that mouse strains quite resistant to skin carcinogenesis by initiation-promotion may be very sensitive to tumor induction by multiple carcinogen treatment. This implies that resistance may be related to poor responsiveness to TPA, the commonly used promoter, and, conversely, sensitivity may be a consequence of high responsiveness to TPA.

Recent studies in our laboratory have provided strong support for a relationship between initiation and resistance to Ca^{2+}-induced terminal differentiation. In direct comparisons of the yield of Ca^{2+}-resistant foci from Balb/c and SENCAR epidermal cells initiated either _in vivo_ or in culture by DMBA or MNNG, little, if any, difference between strains in number of foci obtained for a given treatment has been found. Notable was the frequent presence of foci in untreated dishes of SENCAR but rarely Balb/c cells. This result is consistent with the finding _in vivo_ of papillomas in SENCAR animals treated with promoter alone, in the absence of exogenous initiation. These results suggest the existence of a population of endogenously initiated cells in the SENCAR epidermis. It is not yet clear to what extent these cells may contribute to SENCAR sensitivity.

In spite of the absence of quantitative strain differences in carcinogen-induced, Ca^{2+}-resistant foci, there are qualitative differences which may provide useful clues but have been difficult to quantify. Although some SENCAR

foci resemble those seen in Balb/c cultures, others have a different appearance and behavior. SENCAR foci are often larger than Balb/c foci, and the individual cells in these foci are more easily distinguishable than in Balb/c foci in which the cells appear to be more differentiated, smaller, and more stratified. In general, it has been much easier to obtain cell lines from SENCAR than from Balb/c foci due to differences in survival after subculture. The inducibility of ornithine decarboxylase by TPA and transglutaminase by retinoic acid was generally greater in cell lines derived from SENCAR than from Balb/c foci. The resistant cell lines obtained from SENCAR foci differ in some responses to TPA in culture from cell lines derived from papillomas on SENCAR mice. In particular, papilloma cell lines but not Ca^{2+}-resistant lines appear to be directly stimulated to proliferate by TPA in thymidine incorporation and colony-formation experiments.

Recent in vivo carcinogenesis studies have shown that the kinetics of formation and regression of papillomas in SENCAR and CD-1 mice differ notably. SENCAR papillomas regressed even with continued treatment with TPA while in CD-1 mice the papilloma incidence increased with continued promoter treatment. The TPA-dependent papillomas in CD-1 may represent "dead end" lesions which have no chance of progression to carcinomas. Additional in vivo studies demonstrate that the potential of papillomas in SENCAR skin to become carcinomas is related to the protocol of TPA treatment. Papillomas resulting from shorter (5 wk) periods of promotion are more likely to become malignant than papillomas resulting from longer (20 wk) periods of promotion.

Significance to Biomedical Research and the Program of the Institute:

Epidemiological and medical genetic data have indicated major individual differences in cancer risk in humans. Increased risks are associated both with overall susceptibility to cancer or susceptibility in a particular target organ. In some cases, specific genetic changes have been associated with increased risk, but in many examples, polygenic influences appear more likely. To date, biochemical epidemiological studies have focused only on genetic differences in carcinogen metabolism. In the complex and multistage evolution of cancer, it seems unlikely that carcinogen metabolism is solely responsible for enhanced risks. In fact, it seems likely that factors associated with the expression of neoplastic change would play an important role in host susceptibility. The development of animal strains through selective breeding with high susceptibility at a particular organ site provides an excellent model for the study of susceptibility determinants. In vivo studies have indicated that carcinogen metabolism is unlikely to explain the sensitivity of SENCAR cells, and grafting experiments indicate the target tissue itself is somehow more susceptible. This validates the use of in vitro techniques to explore susceptibility. If this model reflects susceptibility determinants in human cancer, it will provide important insights and potential assays for studies in human populations. Furthermore, understanding determinants of susceptibility is likely to yield information concerning the molecular mechanisms of carcinogenesis.

Proposed Course:

In view of the very short latency period in SENCAR, the high sensitivity to toxic effects of TPA, and the in vitro evidence for essentially equivalent initiation in SENCAR and Balb/c epidermis, it appears that a major role in SENCAR susceptibility is played by promotion.

A recent study (DiGiovanni et al., Carcinogenesis, 5:1493, 1984) suggests that the inbred mouse strain DBA/2 is equally as sensitive as SENCAR when initiated by the directly acting MNNG and promoted by TPA. Use of an inbred strain rather than SENCAR could make possible experiments which otherwise could not be done. We will incorporate DBA/2 mice into some future protocols, as well as inbred SENCAR mice which are now available from Dr. Thomas Slaga at the University of Texas.

We will conduct four types of experiments to assess the importance of constitutively-initiated cells in SENCAR susceptibility: 1) If DBA/2 and SENCAR mice are equally sensitive, we might determine whether a constitutively-initiated cell population also exists in the former, thus providing a clue to whether those cells are important in SENCAR sensitivity. Therefore, we will utilize these mice in experiments using our system to isolate foci of cells resistant to Ca^{2+}-induced terminal differentiation after carcinogen treatment and look for foci in untreated dishes as well as compare dose responses. 2) In other experiments, we will initiate mice in vivo by topical application of an initiating dose of DMBA and subsequently treat with either TPA or solvent with the same protocol used in carcinogenesis experiments. After 4 or 5 weeks of promotion, epidermal cells from these animals will be cultured and selection made for cells resistant to Ca^{2+}-induced terminal differentiation. One would expect that promotion would expand the clones of initiated cells and that each cell could potentially produce a focus after dispersion for culture. Furthermore, use of mice potentially more responsive to promoters, such as SENCAR or DBA/2, should lead to a more pronounced increase in number of foci in promoter-treated groups. In groups not initiated with carcinogen, treatment with promoter should also lead to increased numbers of foci. 3) We will attempt to derive lines of cells resistant to Ca^{2+}-induced terminal differentiation from foci in SENCAR epidermal cell control cultures not treated with carcinogen and test them in vivo in reconstituted skin experiments under silicone domes on athymic nude mouse hosts. These cells will thus be tested in vivo for their ability to form papillomas, to respond to tumor promoters, and to progress to carcinomas. Other cells to be tested in this system will be cell lines derived from papillomas with varying potential to become malignant. 4) Using the same transfection technology being developed to characterize the genes responsible for resistance to Ca^{2+}-induced terminal differentiation, we plan to try to identify the genes responsible for the endogenous population of SENCAR cells resistant to terminal differentiation and compare these with genes identified after initiation with carcinogens. DNA from cells obtained from spontaneous foci will be transfected into Balb/c primary epidermal cells to try to transfer resistance to Ca^{2+}-induced terminal differentiation. DNA from SENCAR papillomas and carcinomas and spontaneous foci will be transfected into NIH 3T3 cells to see whether activated transforming genes can be detected.

Most of the biochemical studies we have done on response to tumor promoters in culture have been after a single treatment with TPA. We will restudy some of these parameters to see how SENCAR, Balb/c, CD-1, and DBA/2 mice respond to multiple treatments with promoters, following protocols similar to those used in vivo. In particular, we will examine induction of ornithine decarboxylase and transglutaminase, cornified envelope formation, EGF binding, and phorbol ester binding as well as growth parameters such as cell number and thymidine incorporation. We will investigate whether there are systemic, possibly immune system, components of promotion that differ between mouse strains by doing contact sensitivity tests using TPA and other promoters to sensitize the abdomens and test the ears for swelling after a week. Although wounding experiments and the UV carcinogenesis studies in SENCAR mice (Strickland, Carcinogenesis 3: 1487, 1982) already suggest that the susceptibility of SENCAR mice is not restricted to promotion by phorbol esters, promoters not competing for phorbol ester receptors will be used to determine whether SENCAR sensitivity is primarily related to this class of compounds.

Publications:

Kawamura, H., Strickland, J. E., and Yuspa, S. H.: Association of resistance to terminal differentiation with initiation of carcinogenesis in adult mouse epidermal cells. Cancer Res. 45: 2748-2752, 1985.

Strickland, J. E., Allen, P. T., Sauder, D. T., Kawamura, H., Fong, M. C., and Yuspa, S. H.: In vitro comparisons of SENCAR and Balb/c primary epidermal cells. Environ. Health Perspec. (In Press)

Strickland, J. E., Jetten, A. M., Kawamura, H., and Yuspa, S. H.: Interaction of epidermal growth factor (EGF) with basal and differentiating mouse keratinocytes. In Murakami, H. (Ed.): Growth and Differentiation of Cells in Defined Environment. New York, Springer-Verlag (In Press)

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05270-04 CCTP

PERIOD COVERED
October 1, 1984 to September 30, 1985

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)
Molecular Mechanism of Action of Phorbol Ester Tumor Promoters

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	Peter M. Blumberg	Research Chemist	LCCTP	NCI
Others:	A. Y. Jeng	Expert	LCCTP	NCI
	T. Nakadate	Visiting Fellow	LCCTP	NCI
	G. Pasti	Visiting Fellow	LCCTP	NCI
	B. Warren	Guest Researcher	LCCTP	NCI

COOPERATING UNITS (if any)
Boston Univ. School of Med., Boston, MA (A. I. Tauber); Stanford Univ., Palo Alto, CA (P. Wender); Wistar Inst., Philadelphia, PA (T. O'Brien)

LAB/BRANCH
Laboratory of Cellular Carcinogenesis and Tumor Promotion

SECTION
Molecular Mechanisms of Tumor Promotion Section

INSTITUTE AND LOCATION
NIH, NCI, Bethesda, Maryland 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
5.67	4.67	1

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The efforts of the Molecular Mechanisms of Tumor Promotion Section are directed at understanding the early events in the interaction of phorbol ester tumor promoters with cells and tissues. Particular attention is being devoted to the analysis of the major phorbol ester receptor, protein kinase C. Novel protocols have been developed for its purification that are more efficient and afford higher yields than is possible with current methods. Preparation of monoclonal and polyclonal antibodies to the receptor is in progress. The role of lipids in reconstitution of the receptor has been characterized in detail. Phospholipids differ in whether or not they can reconstitute, in the amounts required for reconstitution, and in the phorbol ester structure-activity relations of the resultant complex. Diacylglycerols competitively inhibit phorbol ester binding in vitro, consistent with their being the postulated endogenous phorbol ester analogs. Comparison with the homologous phorbol esters yields differences in affinities of 20- to 30,000-fold, depending on the derivative, implying distinct side chain requirements for phorbol esters and diglycerides. As expected from the in vitro assays, treatment of intact cells with phospholipase C to generate diacylglycerol endogenously or the exogenous addition of appropriate diacylglycerols likewise inhibits phorbol ester binding competitively in vivo and induces phorbol ester-like responses. Identification of the enzymatic activity associated with the phorbol ester receptor has made it possible to analyze the coupling between binding and subsequent response. Most mouse skin tumor promoters, structurally unrelated to the phorbol esters, did not activate protein kinase C in vitro. Unsaturated fatty acids at high concentrations did activate, however. Multiple phorbol ester receptors have been implicated in the heterogeneity of phorbol ester responses. The binding characteristics of intact, cultured keratinocytes change from homogeneous to heterogeneous as differentiation proceeds.

PHS 6040 (Rev. 1/84)

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

P. M. Blumberg	Research Chemist	LCCTP	NCI
A. Y. Jeng	Expert	LCCTP	NCI
T. Nakadate	Visiting Fellow	LCCTP	NCI
G. Pasti	Visiting Fellow	LCCTP	NCI
B. Warren	Guest Researcher	LCCTP	NCI
J. Strickland	Research Chemist	LCCTP	NCI
S. Yuspa	Chief	LCCTP	NCI
U. Lichti	Expert	LCCTP	NCI
S. Aaronson	Chief	LCMB	NCI
J. Lacal	Visiting Fellow	LCMB	NCI
S. Srivastava	Visiting Fellow	LCMB	NCI

Objectives:

The early events in the interaction of the phorbol esters with cells and tissues are being characterized. Specific aims are as follows: 1) purification and biochemical characterization of phorbol ester receptors; 2) determination of the role of phospholipids and receptor modification on phorbol ester receptor function; 3) analysis of the interaction of diacylglycerols, the putative endogenous phorbol ester analogs, with the phorbol ester receptors in vivo and in vitro; 4) determination of the activity of phorbol esters, structurally unrelated tumor promoters, and modulators of tumor promotion on protein kinase C activity; 5) characterization of phorbol ester binding and response in intact cells. The major phorbol ester receptor is protein kinase C. This enzyme is postulated to mediate one of the two pathways activated by a large class of hormones for which receptor occupancy is associated with rapid phosphatidylinositol turnover. Several oncogenes may also function, in part, through this pathway. Understanding of the mechanisms of endogenous modulation of the phorbol ester receptors may thus provide insights into both basic biochemical mechanisms and the process of human carcinogenesis as well as to identify biochemical steps suitable for intervention.

Methods Employed:

This Section uses a wide range of techniques to pursue the above aims. Phorbol and derivatives are isolated from natural sources. Semisynthetic derivatives for affinity labeling, structure-activity analysis, and binding studies are prepared and radioactively labeled as necessary. Binding studies are carried out using the ligands and methodology developed by us. Analysis of receptors utilizes both photoaffinity labeling and standard biochemical membrane methodology. The systems analyzed are chosen as optimal for the specific questions being examined. Brain homogenates, because of their richness in receptors, are being used for receptor purification and biochemical analysis. Mouse skin and cultured keratinocytes are being used to dissect subclasses of receptors. Intact cells are being utilized to determine the relationship between receptor occupancy

and biological responses. Importance is placed upon the ability to relate the answers obtained to the biological system of mouse skin promotion and to coordinate effectively exploitation of the systems being studied by the other Sections of the Laboratory.

Major Findings:

For biochemical analysis of the phorbol ester receptors, development of a suitable purification scheme has been a critical objective. Because of our focus on the phorbol esters as tumor promoters in mice, mouse brain was chosen as the starting material. Mouse brains were homogenized in the presence of divalent cation chelators, which caused most of the receptors to remain in the cytosolic, aporeceptor state. Cytosol was prepared by ultracentrifugation and chromatographed on a DE52 column. The DE52 column was found to be a very useful initial step in the purification. It generally gave a 10-fold purification and the material was stable for months in 50% glycerol at -20°. The column was eluted with a gradient containing 0 to 300 mM NaCl in the presence of divalent cation chelators and 20 mM Tris-HCl, pH 7.4 at 4°. Phorbol ester binding and protein kinase activities co-eluted. The ratio of the two activities was constant throughout the peak, which was usually eluted between 60 and 100 mM NaCl. This observation, together with similar results from other laboratories, provided initial evidence that these two activities resided in the same protein entity.

Utilizing the peak fraction from the DE52 column as the starting material, two related purification schemes yielded homogeneous preparations of the receptor. Both took advantage of the impressive capabilities of modern high resolution chromatographic supports. In the first scheme, the DE52 material was run over an anion exchanger Mono Q high resolution column on the Pharmacia fast protein liquid chromatography (FPLC) system. The column was eluted with a gradient containing 0 to 300 mM NaCl in 20 mM Tris-HCl, pH 7.4 at 20° and 0.5 mM each of EGTA and EDTA. The peak protein kinase activity was normally eluted around 60% of the gradient and was pooled and run over the Mono Q column again under the same conditions except that 1 mM ATP was included in all of the buffers. The rationale was that ATP should interact with the protein kinase and shift its chromatographic properties, whereas proteins which do not have kinase activity should be unaffected. Under this new condition, the peak protein kinase activity was eluted at 50% of the gradient. This shift in the elution profile provided a highly enriched protein band with a molecular weight about 82,000 and 2 minor bands as seen in SDS polyacrylamide gels. The minor bands could be removed subsequently by a Biorad TSK phenyl 5PW column. This purification method is fast. About 200 μg of purified receptors could be obtained 2 to 3 days after the DE52 column chromatography. In the second purification scheme, the first Mono Q column was replaced by a Blue Sepharose column with subsequent steps remaining the same. This procedure also generated purified receptor with a similar yield compared with the first scheme. The protein kinase activity, averaged from nine different preparations, was 2.0 ± 0.2 (SD) μmol phosphate transferred/min/mg of protein using Sigma histone IIIS as the phosphate acceptor, and the [^{3}H]PDBu binding activity was 7200 ± 1100 pmol/mg of protein.

A major difficulty in the purification of the receptor and in its subsequent use in analysis was that the purified receptor, in contrast to the partially purified DE52 material, was highly unstable. The major cause of instability appears to

be adsorption to surfaces, which can be minimized by inclusion of very low concentrations of detergent such as Triton X-100. Stored in 0.01% Triton X-100 at -70° for one month, the receptor lost only 20% of its activity.

Polyclonal antibodies have been made to the aporeceptor isolated from mouse brain by the methods we developed and are currently being evaluated. They react with the purified protein in Western blots, and, in the presence of Pansorbin, are able to immunoprecipitate the receptors. They do not, however, directly inhibit either phorbol ester binding activity or protein kinase C enzymatic activity. Monoclonal antibodies are being prepared to the aporeceptor isolated from rat brain. Several positive clones have been identified.

Protein kinase C has been postulated to consist of distinct catalytic and enzymatic domains. Conditions of proteolysis have been established to generate fragments bearing these different domains. Their _in vitro_ properties are currently being characterized and their _in vivo_ activity is being determined by microinjection.

The availability of purified protein kinase C permits analysis of its substrates and confirmation of its role in processes in which it has been postulated to be important based on the responsiveness of the process to phorbol esters. One such process of considerable interest is generation of the oxidative burst in neutrophils. Active oxygen species are implicated in the pathways for a variety of phorbol ester responses. The neutrophil is one of the best studied systems in which active oxygen is generated in response to the phorbol esters and it is that system in which active oxygen generation attains the highest level. In collaboration with Dr. A. I. Tauber, Boston University, we have demonstrated that we can reconstitute the neutrophil respiratory burst in the presence of the neutrophil -membrane fraction, purified protein kinase C, anionic phospholipid, phorbol ester, ATP, and additional cofactors. Current efforts are directed at identifying the relevant targets of protein kinase C in the membrane fraction.

Several indirect lines of evidence had suggested that phospholipids were necessary for the functioning of the membrane-bound form of the phorbol ester receptor. The identification of the cytosolic phorbol ester aporeceptor was technically of major importance because it permitted reconstitution experiments to explore, directly, the phospholipid requirements of the receptor. Considerable evidence suggests heterogeneity in both phorbol ester binding and response. Either multiple receptor proteins exist, of which protein kinase C is only one, or else protein kinase C can be differentially modified. Variation in the phospholipids associated with the aporeceptor represents one attractive mechanism for modification.

Our studies described previously, demonstrate that the nature of the phospholipids associated with the aporeceptor can dramatically affect the binding affinity of $[^3H]$PDBu for the complex. Thus, whereas the K_I of $[^3H]$PDBu for the aporeceptor complex with phosphatidylserine was 0.8 nM, that for the complex with the human red blood cell phospholipids was 22 nM. We have now analyzed the effect of these two different lipid environments on structure-activity relations of the phorbol esters and related compounds. We found that many derivatives showed a comparable difference in affinities between the two complexes. The differences

were much smaller, however, for several of the derivatives. Thus, phorbol 12,13-distearate differed in affinity by only a fraction of 2 and phorbol 12-retinoate 13-acetate differed by a factor of 3 between the two complexes. We conclude that variation in phospholipids does in fact have the potential to explain the variety of receptor properties observed in membrane and intact cell systems.

The high evolutionary conservation of the phorbol ester binding had implied the existence of an endogenous phorbol ester analog, which had maintained a constraint on the binding site over evolution. The ability, using the aporeceptor, to manipulate the lipid phase in organic solvents separately from the protein moiety made it possible to examine the potential activity of lipophilic ligands. Diacylglycerols were of particular interest, given their ability to stimulate the protein kinase C enzymatic activity, as were unsaturated fatty acids, some of which have been reported to be weak tumor promoters. Since endogenous analogs of the phorbol esters are potential candidates as promoters and as regulatory molecules, their identification and the understanding of their control is of high priority.

Because of their insolubility, the diacylglycerols had to be incorporated into mixed liposomes with phospholipid before their affinity for the aporeceptor could be determined. Under such conditions, the diacylglycerol derivative 1,2-diolein reduced the apparent [^{3}H]PDBu binding affinity (K_{app}) without affecting the B_{max}. This shift in affinity could have been the result either of a non-specific perturbation of the lipid environment or else of competition at the phorbol ester binding site. If the inhibition were due to competition, then the K_{app} should obey the relationship $K_{app} = K_d (1 + I/K_I)$. This relationship was in fact obtained. Secondly, the competitive mechanism requires that the diacylglycerol interact with the receptor and that it interact in a stoichiometric fashion. Although the lipophilicity of the diacylglycerol precluded the usual approaches for measuring stoichiometry, we developed a method to detect and quantitate this interaction by determining the shift in the apparent binding affinity of the radioactive phorbol ester in the presence of diolein as a function of the molar ratio of diolein to receptor. The results indicated a stoichiometry of 0.86:1 for D-1,2-diolein, again consistent with diolein being a phorbol ester analog.

Comparison of the relative binding affinities of diacylglycerol derivatives with the corresponding phorbol esters indicated that the phorbol esters were, in all cases, more potent. For the dilaurate and dioleate derivatives, the differences in potency were relatively small, 20- and 80-fold, respectively. In the case of the myristate acetate derivatives the difference was much larger, 3×10^4, reflecting the enhanced potency of the phorbol derivative. These results imply distinct side chain dependencies for the two classes of compounds.

In terms of absolute potencies, the binding affinities of the diacylglycerols reflected their local concentration in the liposomes rather than their nominal, molar concentrations. The K_I for diolein, for example, was approximately 0.12% expressed relative to PS, whereas, expressed as a function of PS concentration, it varied from 1 nM to 10 μM. As with the phorbol esters, the diacylglycerol affinities depended on the identity of the phospholipids reconstituting the aporeceptor, suggesting that modulation in phospholipid association

might regulate kinase activation. This result is of particualr importance, since endogenous diglyceride is thought to be involved in two distinct processes--signal transduction at the plasma membrane and intermediary metabolism at the endoplasmic reticulum. It is not known how the cell distinguishes these two pools. Since these membranes differ markedly in their content of anionic phospholipids, variation in protein kinase C affinity could provide an explanation.

These in vitro studies with the diacylglycerols suggested that the endogenous generation of diacylglycerols in cells by addition of phospholipase C or the exogenous addition of diacylglycerols (of the appropriate lipophilicity to transfer from the aqueous media to the cells) would similarly affect phorbol ester binding and induce similar responses to the phorbol esters.

The effects of phospholipase C treatment were tested in two systems, mouse keratinocytes and GH_4C_1 rat pituitary cells. In the mouse keratinocyte systems, collaborative experiments with Drs. Lichti and Strickland demonstrated that treatment with phospholipase C induced a similar morphological change to that seen with the phorbol esters, inhibited binding of epidermal growth factor, and induced the expression of ornithine decarboxylase and transglutaminase--all responses also seen upon phorbol ester treatment. Consistent with the effects being due to the postulated mechanism, the phospholipase C treatment was shown to generate elevated levels of cellular diacylglycerol. Likewise, the phorbol ester receptor binding affinity was decreased with no change in receptor number, the result expected in the presence of biologically effective diglyceride levels.

In the GH_4C_1 cell system, phospholipase C treatment similarly decreased EGF binding and increased secretion of prolactin, two of the biological responses which also occur upon phorbol ester treatment in these cells. Once again, the affinity of the phorbol ester receptors was decreased with no change in receptor number. In this cell type, phorbol ester treatment leads to an apparent change in the proportion of protein kinase C in the membrane and cytosolic compartments following cellular sub-fractionation. A similar result was obtained upon phospholipase C treatment.

A second approach for elevating cellular diacylglycerol levels is to expose the cells to exogenously added diacylglycerol. In order to be active if added exogenously, the diacylglycerols need sufficient aqueous solubility in order to be able to transfer from the aqueous media to the cell membranes but sufficient lipid solubility in order to partition into the membranes effectively. Derivatives meeting these requirements include glycerol 1-myristate 2-acetate (GMA), 1-oleoyl 2-acetylglycerol (OAG), and 1,2-dioctanoylglycerol (dicaprylin). In NIH 3T3 cells, treatment with GMA, like PMA, inhibited EGF binding and stimulated phosphatidylcholine turnover. In both cases, however, the response was more transient with GMA than with PMA, suggesting rapid breakdown of the GMA. The effect in phosphatidylcholine metabolism is of particular note, because this response has not been observed with hormones which act to elevate diglyceride levels as a consequence of enhanced phosphatidylinositol turnover. The discrepancy may reflect differences in the localization of elevated diglycerides within the cells.

The second system in which we examined the effects of diacylglycerol treatment was that of the mouse keratinocytes. At 250 μM, dicaproin, dicaprylin, and OAG inhibited the specific [^{3}H]PDBu binding 64%, 73%, and 40%, respectively. The same compounds at the same concentration resulted in 39%, 42%, and 41% reduction, respectively, of EGF binding compared to control. The induction of ODC and TG required higher concentrations of the diacylglycerols. At 500 μM, the induction of ODC at 4 hr was 2.5-fold by dicaproin, 1.4-fold by dicaprylin, and 2.2-fold by OAG, compared to a 5.1-fold induction by PMA at 100 ng/ml. The induction of ODC by the diacylglycerols was again very transient, whereas the induction by PMA and by phospholipase C was sustained over several hours. TG was only slightly induced by the diacylglycerols: 1.4-fold by dicaproin, 1.1-fold by dicaprylin, and 1.7-fold by OAG, all at 500 μM, compared to a 5.4-fold induction by PMA at 100 ng/ml.

An important consequence of the identification of protein kinase C as the phorbol ester receptor is that it provides the opportunity to determine whether other tumor promoters may also affect it. At high concentrations, unsaturated fatty acids have been reported to be tumor promoters. We found that unsaturated fatty acids both stimulated protein kinase C enzymatic activity and inhibited phorbol ester binding. The inhibition of binding reflected decreases in both B_{max} and K_D, suggesting more than one mechanism of action. The shift in K_D, like that for the diacylglycerols, obeyed the relationship of $K_{app} = K_D (1 + I/K_I)$. The binding affinity was much lower, however, approximately 15% relative to phospholipid. In vivo studies will be necessary to determine whether the activity of unsaturated fatty acids on protein kinase C is related to their promoting activity. Several other structurally unrelated classes of tumor promoters had no effect on protein kinase C activity. These compounds included anthralin, cantharidin, benzoyl peroxide, and 7-bromomethylbenzanthracene.

A related objective has been to clarify phorbol ester structure-activity relations in an effort to design antagonists to the phorbol esters. As part of that effort, we have begun a collaborative effort with an organic synthesis group at Stanford to determine the effect of structural variations in the phorbol ester backbone on binding affinity. The initial derivatives prepared, phorbol 12,13-dibutyrate derivatives lacking the 5-membered ring, showed measurable but quite low activity (K_d = 140-180 μM) although they possess most of the functional groups postulated to be homologous between the phorbol esters and indole alkaloids. Compounds currently are being synthesized to assess the roles of loss of orientation and rigidity in the overall decrease in activity.

A second class of compounds are triheteroatom-substituted monocyclic derivatives, which again show good overlap with the points of similarity between the phorbol esters and indole alkaloids. The compounds display moderate activity (approximately 200 μM) for inhibition of [^{3}H]PDBu binding. Their biological activity has not yet been determined.

Because of their great relevance to the biological endpoint of tumor promotion, we have been particularly interested in the characteristics of the phorbol ester receptors in mouse skin. We have shown previously that mouse skin particulate preparations possess three classes of binding sites (sites 1, 2, and 3) for

[^{3}H]PDBu with dissociation constants (K_d) of 0.7, 10, and 53 nM; [20-^{3}H]12-deoxyphorbol 13-isobutyrate ([^{3}H]PDB), an inflammatory but relatively nonpromoting analog of the phorbol ester tumor promoters, binds to the same preparations only at the first two classes of binding sites (sites 1 and 2), with K_ds of 7 and 86 nM, respectively. The structure-activity relationships for sites 2 and 3 appear to be different. Whereas the quantitative binding affinities at site 2 correspond well with the inflammatory potencies, the values determined at site 3 appear to correlate better with the promoting activities. The skin particulate preparations contain a heterogeneous population of cells and may additionally be subject to artifacts due to cell disruption. We, therefore, wish to extend the studies to intact keratinocytes and to investigate the effect of their differentiation on binding.

It has been shown that Ca^{2+} concentration is critical in regulating the growth and differentiation of epidermal cells. Basal epidermal cells grow in monolayers with a high proliferation rate and an indefinite life span in culture medium containing low Ca^{2+} concentrations (0.2 to 0.09 mM). When these cells are switched to a medium containing a high Ca^{2+} concentration (>0.1 mM), desmosomes form within minutes, cells vertically stratify, differentiate, and eventually slough from the culture dish. Scatchard analyses of [^{3}H]PDBu, binding both to low and high Ca^{2+} cells, yielded curvilinear plots. The total number of binding sites for [^{3}H]PDBu in high Ca^{2+} cells was larger. It was not possible to analyze the binding data by a 3-site model, as was done in the case of skin particulate preparations, because of the low radioactivity associated with cells exposed to less than 1 nM of [^{3}H]PDBu. The data did not give evidence, however, for a site corresponding to site 1 of the particulate preparations. Using the Ligand Program to analyze [^{3}H]PDBu binding to low Ca^{2+} cells, we found a 2-site fit was significantly better than a 1-site fit. The K_ds of these two classes of binding sites in low Ca^{2+} cells were 5.5 and 100 nM, while the binding capacities (B_{max}'s) for the corresponding sites were 0.9 and 1.7 pmol/mg of protein, respectively. Analysis of the binding parameters for high Ca^{2+} cells was achieved by fixing the affinity for [^{3}H]PDBu at the high affinity site at the same value as that in the low Ca^{2+} cells. [^{3}H]PDB binding data (see below) for low and high Ca^{2+} cells tend to support this assumption. Computer analysis yielded a K_d of 100 nM for the lower affinity [^{3}H]PDBu binding site and B_{max}s of 1.2 and 4.3 pmol/mg of protein, respectively, for the two binding sites in the high Ca^{2+} cells. The ratio of the B_{max} of the low affinity site to the B_{max} of the high affinity site is 1.9 in low Ca^{2+} cells and 3.6 in high Ca^{2+} cells. Therefore, it seems that there was an expression of additional low affinity sites for [^{3}H]PDBu upon switching to medium containing high Ca^{2+}. This expression of [^{3}H]PDBu binding sites was not due to the medium used in the binding assay because there was no difference in the binding parameters when [^{3}H]PDBu binding to high Ca^{2+} cells was assayed in low Ca^{2+} medium. Morphological changes occurred rapidly following high Ca^{2+} switch and became very obvious in 2 hr; binding changes were likewise apparent by 6 hr.

Compared to [^{3}H]PDBu, [^{3}H]DPB bound to keratinocytes with lower affinity, as found in the case of skin particulate preparations. For concentrations of [^{3}H]DPB up to 150 nM, there was only one class of binding site detectable either in low or high Ca^{2+} cells. By competition studies, this [^{3}H]DPB binding site was found to correspond to the higher affinity site for [^{3}H]PDBu binding. Attempts at showing a lower affinity binding site at higher concentrations of

[^{3}H]DPB were not successful due to the increasing amount of non-specific binding. In low Ca^{2+} cells, the B_{max} for [^{3}H]DPB binding was 1.3 ± 0.2 pmol/mg of protein, with a K_d of 69 ± 6 nM. The corresponding values in high Ca^{2+} cells were 1.5 ± 0.4 pmol/mg of protein and 96 ± 26 nM, respectively.

Several Ca^{2+}-resistant cell lines have been developed from carcinogen-treated primary keratinocyte cultures by LCCTP. In high Ca^{2+} medium, these cell lines maintain a high proliferation rate. These cells are not tumorigenic and demonstrate characteristics consistent with their being initiated cells. [^{3}H]PDBu binding to three lines, 308, D, and F, maintained in high Ca^{2+} medium was studied. Scatchard plots showed that cell lines 308 and D had only one class of binding site in high Ca^{2+} medium. The B_{max} and K_d were 1.0 ± 0.1 pmol/mg of protein and 6.7 ± 0.7 nM, respectively, for line 308; and 2.5 ± 0.1 pmol/mg of protein and 11 ± 1 nM for line D. Line F yielded slightly curved Scatchard plots, but failed to give a meaningful fit to a 2-site model. If analyzed with a 1-site model, it yielded binding parameters similar to line D, namely a B_{max} of 2.3 ± 0.1 pmol/mg of protein and a K_d of 9.2 ± 0.7 nM.

We conclude that the state of epidermal differentiation can modulate the amount of the low affinity binding sites for the phorbol esters. Current efforts are directed at determining whether the different sites are coupled to different biological responses.

Significance to Biomedical Research and the Program of the Institute:

Much of human cancer is thought to result from a combination of carcinogenic and tumor-promoting activities. Although considerable progress has been made in elucidating the mechanisms of carcinogens, much less is understood about the mode of action of tumor promoters. One of the most accessible model systems for analyzing this process is that of phorbol ester tumor promotion in mouse skin. The unique value of the phorbol esters in this system lies in their very high potency, which facilitates the distinction between specific and nonspecific effects. The Laboratory of Cellular Carcinogenesis and Tumor Promotion is conducting an integrated study of the skin tumor promotion system at multiple levels of analysis--whole animal, cellular, and biochemical. The focus of the Molecular Mechanisms of Tumor Promotion Section on phorbol ester receptors should identify, unambiguously, the initial biochemical steps which are both necessary and sufficient for tumor promotion by these agents. Identification of such biochemical steps should permit the analysis of their control, modulation, and function in human cells under normal and pathological conditions. Determination of the ability of other less specific tumor promoters to perturb, indirectly, the same processes will shed light on the generality of mechanisms of promotion. Such information is of central importance in attempting to develop better means of detecting tumor promoters and evaluating their potential hazard. In addition, the biochemical analysis should provide both a new avenue for the rational development of inhibitors of promotion as well as shed light on the mechanism of current classes on inhibitors. Particular effort currently is being devoted to the analysis of the major phorbol ester receptor, protein kinase C.

The subdivision of tumor promotion in vivo into multiple stages implies that cellular or biochemical mechanisms (or indeed in vitro assays) will also be

stage-specific. The analysis of functional receptor subclasses provides an essential basis for determining which processes belong to which subclass of response. Moreover, emerging evidence suggests that subclasses of receptors may have antagonistic effects. For example, the tumor yield for the complete tumor promoter, PMA, is reduced by co-administration of first- or second-stage tumor promoters. Understanding of the interaction between the processes belonging to each subclass may be essential for predicting biological outcome and may provide an additional means of intervention.

Proposed Course:

Only part of the total phorbol ester binding activity is found in the cytosol. We plan to characterize the form of the brain receptor which remains membrane-associated in the presence of divalent cation chelators and compare it with the cytosolic aporeceptor. Complementary approaches will be direct purification after detergent solubilization and immunoprecipitation. To better understand the relation of the mouse system to the human, we will purify the aporeceptor from human tissue and prepare antibodies to it. Preliminary experiments indicate that human neutrophils may be a suitable source. Evidence from Nishizuka's laboratory suggests that the regulatory and catalytic domains on protein kinase C may be distinct. We are in the process of generating functional fragments in vitro and characterizing their properties. Biological activity of the fragments will be determined by micro-injection. Complementary studies will determine whether receptor fragmentation occurs in vivo and is affected by chronic phorbol ester treatment. To detect possible relationships between the phorbol ester receptor and oncogenes, antibodies to oncogene products will be screened for cross-reactivity to the receptor. Oncogene products possessing kinase activity will be of particular interest.

Analysis of kinase substrates will focus on proteins thought to be of relevance in transformation and phorbol ester action. Preliminary results indicate that, in vitro, protein kinase C phosphorylates the ras oncogene product, p21, at two sites distinct from the autophosphorylation site of p21. Desmosomes, which form within minutes in keratinocytes subjected to Ca^{2+}-switch, are also phosphorylated. A critical issue will naturally be comparison of in vivo and in vitro phosphorylation behavior, which will be necessary to determine the relevance of observed effects.

The effort to understand the role of lipids and diacylglycerols in receptor function will continue to be pursued vigorously. The state of association of phospholipids with the aporeceptor in intact cells will be probed using photoactivatable phorbol esters such as the phorbol 12-p-azidobenzoate 13-benzoate. Of particular interest will be whether changes in the phospholipid environment are associated with differences in receptor affinity or response. The binding sites for the phorbol esters and diacylglycerols will be identified and compared using derivatives with the carbene-generating photoactivatable side chains 3,3,3-trifluoro-2-diazopropionate and 1-W (m-^{3}H-diazirino) phenoxyundecanoate. Synthesis of the latter compounds has already begun. The possible role of differences in subcellular localization on heterogeneity of response will be explored. Studies will include characterization of the biological effects of the impermeable phorbol esters such as phorbol 12,13-distearate and determination of the reconstitution behavior of the aporeceptor with phospholipid mixtures

corresponding to various intracellular organelles. Of particular interest will be differences between the plasma membranes, the nuclear membrane, and the endoplasmic reticulum for which the lipid compositions have been reported. These studies will be complemented by immunofluorescence to examine receptor localization and shifts in localization upon phorbol ester treatment. Structure-activity relations for phorbol esters and other analogs as a function of lipid environment and ionic conditions will continue to be examined. Attempts will be made to screen tissue preparations for other endogenous modulators of binding and enzymatic activity.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05445-01 CCTP

PERIOD COVERED
October 1, 1984 to September 30, 1985

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)
Molecular Regulation of Epidermal Specific Differentiation Products

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	Dennis R. Roop	Senior Staff Fellow	LCCTP	NCI
Others:	S. H. Yuspa	Chief	LCCTP	NCI
	T. Krieg	Guest Researcher	LCCTP	NCI
	J. Harper	Staff Fellow	LCCTP	NCI
	L. De Luca	Research Chemist	LCCTP	NCI
	F. Huang	Expert	LCCTP	NCI
	P. Steinert	Senior Investigator	DB	NCI
	J. Stanley	Senior Investigator	DB	NCI

COOPERATING UNITS (if any)
Microbiological Asso., Bethesda, MD (E. F. Spangler); Baylor College of Medicine, Houston, TX (James H. Clark); Duke University Medical Center, Durham, NC (Mark S. Kronenberg); Agouron Institute, La Jolla, CA (Cile Blatt)

LAB/BRANCH
Laboratory of Cellular Carcinogenesis and Tumor Promotion

SECTION
In Vitro Pathogenesis Section

INSTITUTE AND LOCATION
NIH, NCI, DCE Bethesda, Maryland 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:

CHECK APPROPRIATE BOX(ES)
☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

cDNA clones that correspond to the major keratin genes expressed in mouse epidermis, the 50-, 55-, 59-, 60- and 67-kd keratin genes, have been isolated and characterized. Several lines of evidence are presented which suggest that the expression of these keratin genes is coordinately regulated and dependent on the state of differentiation. The 50-, 55- and 60-kd keratin genes are mainly expressed in proliferating basal cells and the 59- and 67-kd keratin genes are preferentially expressed in differentiated cells (the suprabasal layers) within the epidermis. This highly regulated program of gene expression was found to be altered by the exposure of mouse skin to agents known to disrupt normal differentiation such as the tumor promoter, TPA. Furthermore, malignant epidermal tumors, which exhibit altered differentiation programs, do not express the differentiation-associated keratin genes. Analysis of amino acid sequence data for keratin subunits, deduced from nucleotide sequence of corresponding cDNA clones, revealed fundamental differences in the primary sequence of keratin subunits expressed at different states of differentiation that may alter the properties and function of keratin filaments containing these subunits. This sequence information has permitted the production of antisera that are monospecific for individual keratin subunits and can discriminate between benign and malignant tumors. To determine if common regulatory sequences are shared by keratin genes that are coordinately induced during differentiation, we have isolated genomic clones for the 59- and 67-kd keratins and determined their nucleotide sequence. In order to facilitate the elucidation of mechanisms regulating the expression of this family of genes, we have developed an <u>in vivo</u> model system (vaginal epithelium) that permits the controlled induction of differentiation and keratin gene expression.

PROJECT DESCRIPTION

Names, Titles, Laboratory, and Institute Affiliations of Professional Personnel Engaged in this Project:

Dennis R. Roop	Senior Staff Fellow	LCCTP	NCI
S. H. Yuspa	Chief	LCCTP	NCI
T. Krieg	Guest Researcher	LCCTP	NCI
J. Harper	Staff Fellow	LCCTP	NCI
L. De Luca	Research Chemist	LCCTP	NCI
F. Huang	Expert	LCCTP	NCI
P. Steinert	Senior Investigator	DB	NCI
J. Stanley	Senior Investigator	DB	NCI

Objective:

To isolate and characterize the genes coding for the major differentiation products of epidermal cells, the keratins. To study the expression of these genes during normal differentiation and during various stages of carcinogenesis.

Methods Employed:

The isolation of keratin cDNA clones is accomplished by the purification of epidermal mRNA, reverse transcription and cloning of double stranded cDNA in the plasmid p-R322. The cDNA clones are characterized by hybridization-selection assays and by direct DNA sequence analysis. Keratin genes are isolated by screening genomic libraries with nick-translated cDNAs and characterized by restriction endonuclease digestion and direct DNA sequence analysis. The expression of specific keratin genes is monitored by RNA blot analysis and quantitated by slot-blot analysis. Monospecific antisera are produced with synthetic peptides corresponding to unique sequences within keratin subunits. The antisera are used to monitor normal and abormal expression of keratin polypeptides by immunofluorescent staining, immunoblotting, and immunoprecipitation.

Major Findings:

The major keratins synthesized by newborn mouse epidermis are 67-, 60-, 59-, and 55-kd. Placing newborn mouse epidermal cells in culture in medium containing low Ca^{2+} results in growth as a monolayer without stratification. These cells proliferate rapidly and display many characteristics associated with basal cells found in intact epidermis. The major keratins synthesized by these cells are 60-, 59-, 55- and 50-kd. In order to isolate cDNA clones for all the major keratins synthesized by epidermal cells, we prepared cDNA libraries from poly (A) RNA isolated from newborn mouse epidermis and primary cultures of mouse epidermal cells. We were able to identify and characterize cDNA clones corresponding to the 67- and 59-kd keratins synthesized *in vivo* and the 60-, 55-, and 50-kd keratins synthesized *in vitro*.

When these cDNA clones are labeled by nick translation with [^{32}P] and hybridized to RNA which was isolated from newborn mouse epidermis and primary cultures of mouse epidermal cells and blotted onto nitrocellulose paper, it is quite evident

that the 59- and 67-kd keratin genes are not expressed at all or only at very low levels in primary epidermal cell cultures compared to newborn epidermis. Transcripts complementary to the cDNA clones isolated from the in vitro library, the 50-, 55- and 60-kd keratins, are present in RNA isolated from intact epidermis but at reduced concentrations compared to those found in cell culture. These results presumably reflect the relative contribution of RNA in basal cells (which consists of a single cell layer) to that of total epidermis (which consists of many cell layers at different stages of differentiation) and indi cate that the 50-, 55- and 60-kd keratin genes are predominantly expressed in proliferating basal cells and that the 59- and 67-kd keratins are predominantly expressed in differentiated cells. These results may also suggest that the stability of transcripts synthesized in basal cells decreases as cells differentiate and leave the basal layer.

Recently, we have obtained two lines of evidence to support this hypothesis. First, as discussed later, we have been able to produce antisera that are monospecific for the 55-, 60-, 59- and 67-kd keratin subunits using synthetic peptides corresponding to unique sequences located at their C-termini (sequence data will be summarized below). These antisera have been used to localize these subunits within newborn mouse epidermis by indirect immunofluorescence. The 59- and 67-kd subunits were only present within the differentiated cells of the epidermis (the suprabasal layers) and not in the (the basal layer). The 55- and 60-kd subunits, however, were detected in both the basal and suprabasal layers. Second, we have recently been able to detect mRNA coding for different keratin subunits within cells in different layers of the epidermis by in situ hybridization to histological sections of newborn mouse skin. We have subcloned the keratin cDNAs into newly developed vectors that permit the synthesis of RNA transcripts that can be labeled with [^{35}S] using [^{35}S]-uridine 5'-(α-thio) triphosphate. Insertion of the cDNAs into the vector in the correct orientation and subsequent transcription results in the synthesis of transcripts that are complementary to mRNA. In our initial experiments, we have been able to localize the majority of the mRNAs coding for the 55- and 60-kd keratins to the basal layer of the epidermis. Very little of these mRNAs is found in the differentiated cell layers, suggesting that expression of these keratin genes is "turned off" as cells differentiate and migrate into the suprabasal layer. Just the opposite was observed for mRNAs for the 59- and 67-kd keratins, i.e., these mRNAs were predominantly localized within the differentiated suprabasal layers and not the basal layer.

On the basis of these combined data, we believe that the expression of specific subsets of keratin genes is correlated with the state of differentiation and we consider expression of the 50-, 55- and 60- kd keratin genes to be associated with proliferation and expression of the 59- and 67-kd keratin genes to be associated with differentiation.

Considerable evidence has been accumulated which suggests that the tumor promoter, TPA, can alter the normal program of epidermal differentiation. Since the regulation of keratin gene expression is tightly controlled during differentiation, it was of obvious interest to see what effects TPA would have on expression of these genes. A dramatic decrease in the level of the differentiation-associated keratin mRNAs to approximately 10% of control epidermis was observed within 12 h after exposure to TPA. In contrast, the transcript levels of the proliferation-

associated keratin genes were elevated. The decrease in mRNAs corresponding to the differentiation-associated keratins probably reflects an acceleration of terminal cell maturation which is mediated by TPA. Recently we have been able to show that this decrease occurs within 4 h after exposure to TPA. This rapid decrease in mRNA levels suggests that a decrease in mRNA synthesis is also accompanied by an increase in rate of degradation of these mRNAs. The order of recovery of the differentiation-associated keratin mRNAs was reversed as compared to that induced in vaginal epithelium by estrogen, a normal inducer of differentiation to be discussed later. This may indicate that the coordinated program of keratin gene expression observed during normal differentiation is uncoupled when the transit time from the basal to upper differentiated layers is decreased. The limited effect on mRNA levels of the proliferation-associated keratins indicates that the initial increase in keratin mRNA instability is restricted to the differentiated cell layers. The increase in the proliferation-associated keratin mRNAs may result from expansion of the basal cell compartment due to the regenerative hyperplasia which follows TPA treatment. Similar results were also obtained for skin exposed to weak tumor promoters or hyperplasiogens such as 4-O-methyl-TPA, A23187 (a Ca^{2+} ionophore) and mezerein at concentrations which produce hyperplasia. These results provide evidence at the molecular level to support the conclusions, based on other data, that tumor promoters accelerate the rate of differentiation in a subpopulation of mouse epidermal cells and directly or indirectly induce another subpopulation of cells to proliferate.

On the basis of polyacrylamide gel electrophoresis, cytoskeletal extracts from carcinomas consistently did not contain the 67-kd keratin subunit, whereas papillomas did. We wanted to determine if the failure of carcinomas to synthesize the 67-kd keratin was due to their inability to translate existing mRNA for the 67-kd keratin gene or due to the lack of expression of this gene. Therefore, we compared the level of expression of keratin genes in carcinomas and papillomas with that of control adult epidermis by slot blot analysis. Transcript levels for keratin genes are very similar in papillomas and normal adult epidermis with the exception of an increase in the transcript level corresponding to the 55-kd subunit. In contrast, transcript levels of the differentiation-associated keratin genes are very low in carcinomas, whereas transcript levels of the proliferationassociated keratin genes are less altered. Thus, the absence of the 67-kd keratin subunit in carcinomas is due to their failure to express this gene. Moreover, these tumors fail to express the other differentiation-associated keratin genes as well. These observations prompted us to develop an assay, which would distinguish between benign and malignant tumors to be described below.

The determination of the primary sequence of keratin proteins by classical protein sequencing techniques has not been feasible due to their insolubility. The availability of keratin cDNA clones provided the first opportunity to determine the amino acid sequence of these proteins. In collaboration with Dr. Peter Steinert, we have deduced the complete amino acid sequence of the 59- and 60-kd keratins and approximately two-thirds of the sequence of the 50-, 55- and 67-kd keratins from the nucleotide sequence of their cloned cDNAs. This sequence information has enabled us to make several fundamental observations concerning the subunit structure of keratin proteins and to formulate a hypothesis explaining the requirement for expression of a new subset of keratin genes during terminal differentation. In addition, this sequence information allowed us to

determine that the C-terminal residues were unique for different keratin subunits. This observation was exploited to produce antisera that were monospecific for individual keratin subunits which will be described below.

In collaboration with Dr. Peter Steinert (Dermatology Branch, NCI), we have isolated and sequenced the mouse 59-kd keratin gene and the human 67-kd keratin gene. This project was initiated in order to determine the genomic organization of these keratin genes and to identify common regulatory sequences that may be shared by genes that are coordinately expressed during differentiation. These genes were isolated by screening mouse and human genomic libraries with the appropriate cDNA clones. We were able to determine that the 59-kd keratin gene contained 7 introns and the 67-kd keratin gene contained 8 introns. It was of interest to determine if the introns present within these keratin genes occur at locations delineating functional domains as has been observed for several other eukaryotic structural genes. Most of the introns present within these genes occur at similar positions within the region encoding sequences predicted to form coiled-coils, but do not delineate obvious structural subdomains. Interestingly though, most of the introns are interrupted at or near the beginning of the characteristic seven residue (heptad) repeat of sequences which form the coiled-coil. These data suggest that these genes arose from a common ancestor which may have been assembled from smaller units containing multiple heptad repeats. In collaboration with Dr. Cila Blatt, we have recently found by restriction fragment length polymorphism analysis that two mouse keratin genes, the 60-kd and 67-kd keratin genes, are located on chromosome 15 and that they are linked.

As discussed above, all keratin subunits contain a central domain of about 300 residues which form a coiled-coil α-helical structure. The production of antisera that are monospecific for individual keratin subunits has been difficult due to the presence of common antigenic determinants within this conserved region. A comparison of available sequences for keratin subunits revealed that the carboxy-terminal sequences were unique with the exception of the 55- and 50-kd subunits which have identical C-terminal residues. This finding, coupled with a previous observation that these C-terminal sequences have a peripheral location within filaments, encouraged us to try to elicit specific antibodies for keratin subunits with synthetic peptides corresponding to the C-terminal sequences. Our initial attempt used synthetic peptides for the residues of the 59- and 67-kd subunits. The antisera produced against these synthetic peptides was highly specific for the appropriate keratin subunit as judged by immunoblot analysis. This same approach was then used to produce antisera that were monospecific for the 55- and 60-kd subunits.

These antisera have been used to localize these keratin subunits within newborn mouse skin by indirect immunofluorescence. The 59- and 67-kd subunits were only present within the differentiated cells of the epidermis (the suprabasal layers) and not in the proliferating cells (the basal layer); in contrast, the 55- and 60-kd subunits are detected within the basal and suprabasal layers. These results lend additional support to the contention that the expression of certain subsets of keratin genes is correlated with the state of differentiation.

Since we had previously shown that the concentrations of messenger RNAs for the differentiation specific keratins were greatly reduced in carcinomas as compared to papillomas and normal adult epidermis, we were eager to see if this difference in expression could be detected at the protein level using the monospecific keratin antisera. Cytoskeletal extracts were prepared from individual papillomas (induced in female SENCAR mice by initiation with DMBA (20 µg) followed by promotion with TPA (2 µg twice weekly for 12 weeks)) and from individual carcinomas (derived from papillomas induced as above but subsequently treated with urethane (20 mg i.p. once weekly) to accelerate malignant conversion and assayed by immunoblot analysis. Polypetides corresponding to the 55- and 60-kd keratin subunits were detected in extracts from both papillomas and carcinomas as expected from data obtained at the mRNA level. Also, consistent with the mRNA levels, papillomas contained the 59- and 67-kd keratin subunits, whereas carcinomas did not. In addition to the immunoblot analysis data, we have been able to distinguish between papillomas and carcinomas by indirect immunofluorescence staining of biopsies with antisera specific for the 59- and 67-kd keratins.

In collaboration with Dr. Freesia Huang (LCCTP) and Dr. Luigi De Luca (LCCTP), we have used these specific antisera to examine changes in the pattern of keratin synthesis in hamster tracheal epithelium after induction of squamous metaplasia. To summarize briefly, synthesis of the 60-kd keratin does not occur in normal tracheal epithelium. However, this keratin subunit can be readily detected by immunoblot analysis or indirect immunofluoresence with the monospecific antiserum for the 60-kd keratin after the induction of squamous metaplasia by vitamin A deficiency or exposure to a chemical carcinogen. These results suggest that synthesis of the 60-kd keratin may be a marker for the metaplastic changes which occur in tracheal epithelium as a result of vitamin A deficiency or during intermediate stages of carcinogenesis. The availability of antibodies which react specifically with this keratin provides a convenient assay for these changes.

To study the mechanism by which differentiation and consequently the expression of specific keratin genes is regulated, it is desirable to have a system in which induction of the differentiation process is easily controlled. The epidermis is not well suited for this type of study since it is difficult to manipulate the differentiation process in a controlled manner. Vaginal epithelium is an attractive alternative to the epidermis since the state of differentiation of this tissue is hormone dependent and can be easily changed in ovariectomized animals by administering exogenous hormone. In addition, the differentiation state of this epithelium has also been shown to be very sensitive to retinoids. Changes in the pattern of keratin gene expression have therefore been used to examine the effects of estrogen and vitamin A on the differentiation program of vaginal epithelium.

The differentiation program induced by estrogen in rat vaginal epithelium is similar to that of the epidermis histologically; therefore, it was of interest to determine if the same keratin genes, which have been studied extensively in the epidermis, were also expressed in vaginal epithelium. Initially we isolated the cystoskeletal proteins expressed in vaginal epithelium of ovariectomized rats 48 h after exposure to estradiol. A comparative immunoblot analysis, employing a multivalent antiserum produced against mouse keratins, indicated that the

keratins produced in both tissues were similar. Therefore, a more stringent assay was performed for identity of molecular hybridization of [^{32}P]-labeled mouse cDNA probes to estrogenized rat vaginal RNA that had been separated under denaturing conditions on an agarose gel and transferred by blotting onto nitrocellulose paper. Using identical hybridization conditions, rat vaginal epithelium was shown to contain transcripts for the 50-, 55-, 60-, 59-, and 67-kd keratin genes that were identical in size to those detected in mouse epidermis. These results indicate that the same keratin genes expressed in mouse epidermis are also expressed in rat vaginal epithelium when an estrogen-induced change in the differentiation program occurs.

The conservation in sequence homology of the keratin genes between the two species allowed us to use the mouse keratin cDNA probes to monitor the hormone induced expression of keratin genes in rat vaginal epithelium. This was most conveniently done by RNA slot blot analysis. Total RNA was isolated from vaginal epithelium obtained from ovariectomized animals at 0, 24 and 48 h after exposure to a single injection of estradiol and analyzed. The very low level of expression of both the proliferation-associated (the 50-, 55- and 60-kd) and the differentiation-associated (the 59- and 67-kd) keratin genes in control vaginal epithelium is consistent with the lack of specialization detected histologically. There is a dramatic induction of the proliferation-associated keratin genes within 24 h after exposure to estradiol and this correlates with the onset of proliferation. The decrease in the level of transcripts of these genes observed at 48 h suggests that the synthesis of these RNAs ceases in differentiated cells and a dilution effect occurs due to the concentration of differentiated cells present at this time. A decrease in the stability of these mRNAs, analogous to that thought to occur in differentiated epidermal cells, may also occur in vaginal epithelial cells and implies additional regulation at a post-transcriptional level.

Induction of expression of the differentiation-associated genes is also observed 24 h after exposure to estradiol; however, transcripts of these genes continue to accumulate at 48 h. The continued synthesis of these differentiation-associated genes at 48 h is consistent with the pronounced stratification and differentiation observed histologically. The induction of these genes also appears to occur sequentially in that induction of the 59-kd keratin gene appears to occur first, followed by the 67-kd keratin gene. These results establish firmly that the induction of expression of these keratin genes occurs at the level of transcription, since only very low levels of transcripts were found in the uninduced epithelium. As was observed in the epidermis, the expression of different subsets of keratin genes in vaginal epithelium is also correlated with the state of differentiation.

Vaginal cornification (i.e., the appearance of cornified epithelial cells in vaginal smears) has been used experimentally as an indication of vitamin A deficiency for many years. It was of interest to determine if the differentiation program induced by vitamin A deficiency was similar to that induced by estrogen as judged by histological examination and by the pattern of keratin expression. The morphology of vaginal epithelium isolated from ovariectomized vitamin A deficient animals is very similar to that observed 48 h after treatment of ovariectomized animals with estradiol. The pattern of keratin synthesis, assayed by immunoblot analysis, is also very similar and in general showed a high level of

the differentiation-associated keratins. Intravaginal application of retinoic acid ($1x10^{-7}$ mole/animal) reversed the morphological appearance of this epithelium to that of a simple unstratified epithelium similar to that observed in control ovariectomized animals. A decrease in the concentration of the differentiation-associated and proliferation-associated keratins on immunoblots was correlated with this morphological change. These results suggest that the effects of vitamin A on the differentiation program of vaginal epithelium are opposite to those observed by estrogen. In addition, the reprogramming appears to occur in the basal layer since all of the differentiated cells in vaginal tissue from vitamin A deficient rats slough from the epithelium when vitamin A is administered.

Significance to Biomedical Research and the Program of the Institute:

The isolation and characterization of cDNA clones corresponding to the major keratins expressed in mouse epidermis have allowed us to establish that the expression of specific subsets of keratin genes is highly correlated with the state of differentiation. These cDNA clones also permitted us to determine the amino acid sequence for these keratins, which was previously not feasible due to insolubility of these proteins. On the basis of this sequence information, we have proposed a general model for keratin subunit structure and suggested that sequence differences observed for keratins expressed at different states of differentiation may change the properties and functions of the filaments that they form. These cDNA clones have been used as very sensitive probes to demonstrate changes in the normal differentiation program that occur at different stages of tumor development during experimental skin carcinogenesis. On the basis of this expression data, we have developed an assay using antisera that are monospecific for individual keratin subunits that can discriminate between benign and malignant epidermal tumors. The ability to distinguish between benign and malignant tumors and possibly detect metaplastic changes that may occur during intermediate stages of carcinogenesis with antisera of such specificities demonstrates the potential usefulness of these reagents not only in the study of various stages in tumor development but also in the clinical histodiagnosis of cancer. The sequence information determined for genomic clones of keratins co-expressed during normal differentiation, but not in malignant tumors (the 59- and 67-kd), may eventually allow us to determine if the lack of expression of these genes is due to changes at the gene level which were induced during carcinogenesis. Ultimately, we would like to determine the mechanism(s) regulating the expression of these genes. The achievement of this goal should provide insight into the mechanism(s) regulating the normal differentiation process and alterations that occur during carcinogenesis. The ability to induce changes in the differentiation program of an epithelium in a controlled manner is a prerequisite for elucidating factors that not only regulate this process but also the expression of genes that result as a consequence. The demonstration that both estrogen and retinoic acid can modulate the differentiation program of vaginal epithelium and keratin gene expression should facilitate the elucidation of the mechanisms regulating the expression of this family of genes.

Proposed Course:

The progress made to date in determining the structure of different keratin genes should enable us to determine if keratin genes that are coordinately induced during terminal differentiation share common regulatory sequences. At the present time, we have compared sequences that are approximately 150 nucleotides upstream from the site of transcription initiation of the mouse 59-kd keratin gene and the human 67-kd keratin gene and have not observed conserved sequence other than consensus sequences for CAT-boxes and TATA-boxes present in all eukaryotic genes. We are presently sequencing farther into the 5' flanking regions of these genes to continue the search. The coding sequences of keratin genes have in general been highly conserved between species throughout evolution and we suspect that common regulatory sequences will also be conserved. However, to eliminate this potential problem we have recently isolated the mouse 67-kd keratin gene and have made considerable progress in mapping this gene in order to begin sequencing.

Another approach that we are taking to identify regulatory sequences involves an attempt to find "enhancer" elements. These sequences can greatly increase the efficiency of transcription of genes and in many cases have been found to be tissue specific. To facilitate our search for these sequences, we plan to screen for efficient expression of the neomycin-resistance gene. We have been able to show that several epidermal cell lines serve as good recipients for the pSV2neo gene by transfection experiments. The SV2neo vector contains enhancer sequences from SV40 to ensure efficient expression. We have deleted these sequences and find that no resistant foci result after transfection with this vector and selection for neo-resistant colonies. We are currently inserting different regions of the 59-, and 67-kd keratin genes into this vector to see if we can identify a region that will substitute for the SV40 enhancer and confer neo-resistance on the recipient cells.

Defining the molecular basis for the failure of malignantly transformed epidermal cells to express the differentiation-associated keratins has a high priority. Although it is quite likely that this lack of expression can be attributed to a failure of these cells to synthesize appropriate regulatory molecules required to induce transcription, we will perform experiments to determine if alterations have occurred at the gene level. The most straight forward approach that would detect gross rearrangements would be restriction mapping and Southern blotting with control DNA. A more detailed analysis would require the isolation of these genes from transformed cells to compare their sequence with that which we have determined for the normal genes. We also want to determine the effect of expression of the differentiation-associated keratin genes on malignantly transformed keratinocytes that do not respond to normal differentiation signals (i.e., Ca^{2+}). We have postulated on the basis of sequence data, that filaments formed from keratin subunits expressed in terminally differentiating cells are likely to have very different properties from the filaments produced in proliferating basal cells (e.g., these filaments may be more rigid and interfere with replication machinery). We are in the process of placing cDNA inserts which contain all of the coding sequences for the 59- and 67-kd keratins into expression vectors that will permit the efficient expression of these DNAs in mouse cells _in vitro_. One of the expression vectors contains the strong promoter in

the LTR of RSV, which was constructed by Dr. Bruce Howard of LMB, NCI. The other expression vector was constructed by Dr. Gordon Hager (LEC) and contains the promoter region from MMTV. The advantage of this system is the fact that this promoter is regulated by glucocorticoids and therefore the expression of genes introduced into this vector can be regulated. Both of these genes will be introduced simultaneously with the neomycin resistance gene (pSV2neo) into a malignant cell line by DNA transfection. We can monitor the uptake of these DNAs by Southern blotting and the expression by RNA slot-blot analysis. Effects of these introduced genes will be followed by monitoring the growth and morphology of the recipient cells.

Initial results with the monospecific antisera suggest that we can use this assay to discriminate between benign and malignant tumors; therefore, it is important to determine if we can use these antisera to detect the conversion of papillomas to carcinomas at stages earlier than those detectable by gross or histological examination. Biopsies are currently being taken at two week intervals from papillomas that were produced by initiating female SENCAR mice with DMBA (20 μg) followed by promotion with TPA (2 μg twice weekly for 12 weeks) and treatment with urethane (20 mg i.p. once weekly) to accelerate malignant conversion. The biopsies will be screened by indirect immunofluorescence with the 59- and 67-kd antisera to determine if the early appearance of areas which fail to stain with the antibodies is indicative of malignant conversion. We also are planning to screen biopsies of papillomas which are being produced by a short promotion protocol which results in a high percentage of the papillomas converting to carcinomas. If we are able to detect the conversion of papillomas to carcinomas at an early stage, we will be able to obtain a more accurate estimate of the potency of genotoxic agents, such as urethane or 4-NQO, used to accelerate malignant conversion and to determine the risk for conversion in benign lesions. In addition, this method of screening will allow us to assay for early changes at the molecular level, such as oncogene expression levels or activation, that may be associated with malignant conversion.

The unique features described for vaginal epithelium establish this as a model system for _in vivo_ studies concerning the induction of differentiative and keratin gene expression. We would like to determine if keratin genes are regulated by a direct interaction of hormone-receptor complexes with regulatory regions upstream from these genes. Although keratin gene expression may be induced as an indirect consequence of hormone-induced differentation, it is possible that the expression of these genes is directly regulated by estrogen. A sophisticated _in vitro_ expression system would be required to prove this. However, sequence information generated for genomic clones described earlier may give us some clues. To date, most of the genes which are regulated by interaction of steroid receptor complexes with promoter regions have been shown to contain similar sequences in this regulatory region. We will be able to scan the 5' flanking sequences of the keratin genes for such consensus sequences. If candidates are found, we can substitute these regions into expression vectors and demonstrate by _in vitro_ mutagenesis and deletion experiments that these sequences are required for steroid induced expression in an appropriate cell line.

The exposure of prenatal or neonatal mice to DES induces a permanent proliferation and keratinization of vaginal epithelium that culminates in hyperplastic

lesions and carcinogenesis. It would be of interest to determine if exposure to DES results in changes in the organization of keratin genes since they appear to be locked in a "transcriptionally on" configuration. Gross rearrangements in gene structure could be determined by Southern blot analysis of restriction digests of control and treated DNAs. The detection of more subtle changes would require the isolation and sequence analysis of these genes. Changes in the chromatin structure around these genes may also have occurred after exposure to DES since these genes are persistently expressed. These changes could be detected by analysis for DNase hypersensitive sites. In addition, the permanent changes in proliferation and differentiation of vaginal epithelium make this an attractive system to assay for changes in the expression and/or activation of known oncogenes that may affect these processes.

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ANNUAL REPORT OF

THE LABORATORY OF CHEMOPREVENTION

NATIONAL CANCER INSTITUTE

October 1, 1984 through September 30, 1985

The problem of the isolation, characterization, and biological role of transforming polypeptide growth factors (TGFs) continues to be the major focus of our laboratory. Previously, we had shown that TGFs can be isolated from a variety of epithelial and mesenchymal tumors of murine, chicken, and human origin, caused either by chemicals or viruses, or of spontaneous origin. All of these TGFs are acid-stable, low molecular weight peptides, that are the subject of current attempts at amino acid sequencing. New methods to achieve purification have been developed in our Laboratory, and we have completed the total purification of TGF-beta from three non-neoplastic tissues. These tissues are human placenta, human blood platelets, and bovine kidney. The experimental use of TGF-beta in wound healing has been a finding of major importance and has provided a great deal of encouragement to proceed further with the entire problem of the molecular biology and molecular genetics of these growth factors. Finally, we are now involved in a major attempt to integrate studies of retinoids into our current program of studies on peptide growth factors.

The present activities of the Laboratory are devoted almost exclusively to studying the chemistry and biology of both type alpha and type beta transforming growth factors. These studies include the interactions of these peptide growth factors with the genetic apparatus of the cell, particularly oncogenes, as well as their interactions with low molecular weight regulatory agents, such as retinoids and steroids. The Laboratory is involved with the total spectrum of studies that can be done with growth factors, ranging all the way from mutating their chemical structure with the most advanced techniques of recombinant DNA research to evaluating their potential therapeutic usefulness as clinical agents in patients with defective wound healing.

We have characterized and purified to homogeneity a new peptide growth factor called transforming growth factor-beta (TGF-beta). Although this peptide was named for its ability to cooperate with members of the epidermal growth factor family to induce phenotypic transformation and anchorage-independent growth of non-neoplastic fibroblasts, we have recently shown that TGF-beta can inhibit the anchorage-independent growth of certain tumor cells. This bifunctional character of TGF-beta is best shown in experiments using fibroblasts transfected with the *myc* gene; in these cells TGF-beta can either stimulate or inhibit the anchorage-independent growth of the cells depending on the complete set of other growth factors operant on the cells.

Human platelets are a major storage site for type beta transforming growth factor; they contain 100-1000-fold more of this peptide than do other cells which have been examined to date. TGF-beta can be purified from platelets in a two-step procedure that involves sequential gel filtration in the absence and then presence of denaturant. Structural studies on homogeneous TGF-beta show that it is composed of two 12,500-dalton subunits which are held together by disulfide bonds. Platelets also contain smaller amounts of an EGF-like

peptide which can synergize with platelet TGF-beta to induce growth of NRK fibroblasts in soft agar. Mechanistically, these two peptides interact; incubation of purified TGF-beta with NRK cells specifically increases the number of receptors for epidermal growth factor.

The complete amino acid sequences for human TGF-alpha and TGF-beta have been deduced from the cDNA nucleotide sequence for each of these peptides cloned in collaboration with Genentech, Inc. Each of these peptides is synthesized as a part of a larger precursor molecule, and the messenger RNAs encoding each of these peptides are considerably larger than expected. TGF-alpha, a single-chain peptide of 50 amino acids, has been expressed in milligram quantities in E. coli; the recombinant peptide is fully active biologically. TGF-beta is a homodimer with each chain composed of 112 amino acids containing 9 cysteine residues; it has not yet been expressed in a biologically active form.

The human cDNA clones for both TGF-alpha and TGF-beta have been used for Northern blot analysis of total cytoplasmic RNA from several human tumor cell lines. The human TGF-beta cDNA clone can be used to detect TGF-beta mRNA in retrovirus-transformed rodent cell lines. The human, rat, and mouse TGF-beta mRNAs are of similar molecular weight (2.4 kb) on denaturing gels. Cloning of the rat TGF-beta gene will both permit direct comparison with the human gene and provide a high stringency probe to investigate mechanisms of transformation of rodent cell lines.

We have identified a single high affinity binding site for TGF-beta on NRK fibroblasts, indicating that both the positive and negative growth regulatory effects of TGF-beta on this cell must be mediated through the same receptor. Every cell type assayed to date possesses a similar high affinity receptor for TGF-beta, which appears to be constitutively expressed under all conditions, since none of the agents that modulate TGF-beta action appear to affect the receptor, and TGF-beta itself causes only a partial down-regulation of the receptor. The receptor appears to be a high molecular weight monomeric protein which, unlike other growth factor receptors, does not undergo ligand-induced clustering or phosphorylation.

The agar colonies induced by the TGFs are tightly associated spherical colonies. Transformed cells, however, often form agar colonies that are loose aggregates of individual cells. If transformed cells release ectopic peptides that contribute to their expressed phenotype, one might expect to find one that can modulate the morphology of agar colonies. The characterization of such activities will hopefully help to define the role of these factors in the initiation, development and spread of malignancies in vivo. Selected agar colonies were cloned from a human melanoma line. The colonies from these clones expressed morphologies ranging from small and tight to large and loose. The effects of these serum free conditioned media (SFCM) were examined; they stimulate the indicator cells to form agar colonies with morphologies similar to the melanoma clones from which the SFCM were obtained. The SFCM from a clone of the melanoma line that produces large loose agar colonies, contains an activity with an apparent M_r of approximately 22 kd that can modulate the tight spherical colonies induced by the TGFs to form looser scattered colonies. These findings indicate some transformed cells are producing factors that can induce analogous behavior in untransformed indicator cells; this suggests the neoplastic phenotype expressed by a transformed cell may be partially determined

by the concentrations and the ratios of the ectopic factors it is releasing. Several mutations have been introduced into a cloned human TGF-alpha gene by site-directed mutagenesis. These mutants will be expressed to study structure-activity relationships of TGF-alpha. The rat TGF-alpha gene has been chemically synthesized for further in vivo studies in rats. The rat TGF-alpha gene will be inserted in a vaccinia virus vector and then used to immunize rats against TGF-alpha activity. An anti-sense rat TGF-alpha gene has been constructed using a retrovirus vector and will be used in an attempt to inhibit specific expression of TGF-alpha.

Retinoid-deficient quail embryos have been used to begin investigation into the interactions of retinoids, growth factors, and oncogenes in control of embryonic development. We have shown that a variety of retinoids are able to restore normal development of the vascular system to retinoid-deficient quail embryos. Reversal of the developmental defects of deficiency has also been demonstrated in in vitro culture of deficient quail blastoderms. Detailed morphological analysis of the defects in development of the heart of the vitamin A-deficient embryo has been carried out with the hope that future use of in situ hybridization techniques will permit analysis of specific alterations at the level of gene expression.

An important area for potential application of peptide growth factors is the enhancement of wound healing. Despite the need for rapid healing in the treatment of severe burns, trauma, diabetic and decubitus ulcers, and other conditions, there is no practical way at present to accelerate wound healing with pharmacological agents. In this project, we have shown that subcutaneous injection of transforming growth factor-beta into the back of rats enhances wound healing, as measured by increased accumulation of total protein, collagen, and DNA. Nanogram amounts of TGF-beta cause a pronounced fibrotic reaction when injected subcutaneously into newborn mice. In other studies with newborn mice, we have shown that human TGF-alpha is as potent as human EGF or mouse EGF in eliciting a physiological eyelid-opening response. All of the above studies indicate that transforming growth factors have potential clinical utility for enhancement of specific growth of cells.

In summary, the Laboratory of Chemoprevention is currently involved in some very new approaches to the control of the growth of cancer cells. These approaches have led to the discovery of new growth factors which themselves may be useful therapeutic agents, as well as to current attempts to synthesize "fraudulent" growth factors or to utilize other mechanisms to antagonize the effects of undesirable growth factors in the cancer cell. Considering the progress that has been made in the past five years, one may be cautiously optimistic that this area of investigation will continue to provide significant results.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05051-07 LC

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Mechanism of Action of Type Beta Transforming Growth Factor

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	Anita B. Roberts	Staff Scientist	LC	NCI
Others:	Mario A. Anzano	Visiting Scientist	LC	NCI
	Paturu Kondaiah	Visiting Fellow	LC	NCI
	Sonia B. Jakowlew	Staff Fellow	LC	NCI
	Joseph M. Smith	Biologist	LC	NCI
	Nannette B. Roche	Biologist	LC	NCI

COOPERATING UNITS (if any)

Massachusetts Institute of Technology, Cambridge, Mass. (R. A. Weinberg and D. F. Stern)

LAB/BRANCH

Laboratory of Chemoprevention

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
5.5	3.5	2

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The purpose of the project is to determine the mechanism of action of type beta transforming growth factor (TGF) both in cell culture *in vitro* and in animal studies *in vivo*. Particular emphasis will be placed on identification of the switching mechanisms active in control of the bifunctional responses of cells to TGF-beta, or, stated differently, on determination of the particular gene expression which leads, on the one hand, to stimulation of cell proliferation by TGF-beta, and, on the other hand, to inhibition of proliferation by TGF-beta. Thus far, three different classes of molecules have been found to influence the response of cells to TGF-beta; these include 1) other polypeptide growth factors such as TGF-alpha, epidermal growth factor, and platelet-derived growth factor; 2) retinoids and other low-molecular weight effectors; and 3) oncogenes and their polypeptide products. Each of these classes of substances is known to affect gene expression. Tools that will be used to assess TGF-beta function in this project will include specific bioassays, receptor assays, immunoassays, and assays for TGF-beta messenger RNA. Studies are aimed at two levels: in the whole animal and in cell lines in culture. Specific systems employed for these investigations include 1) an experimental model in the newborn mouse for studying the effects of TGF-beta on induction of a granulation-type response to injury; 2) effects of TGF-beta on matrix proteins of specific cell types thought to contribute to the observed fibrotic effects *in vivo*; and 3) study of the modulation of TGF-beta effects brought about by transfection of various oncogenes into cell lines responsive to TGF-beta.

PHS 6040 (Rev. 1/84)

POSITION DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

Anita B. Roberts	Staff Scientist	LC	NCI
Mario A. Anzano	Visiting Scientist	LC	NCI
Paturu Kondaiah	Visiting Fellow	LC	NCI
Sonia B. Jakowlew	Staff Fellow	LC	NCI
Joseph M. Smith	Biologist	LC	NCI
Nannette B. Roche	Biologist	LC	NCI

Objectives:

This project is directed towards understanding the mechanisms controlling the bifunctional effects of TGF-beta on cells and as such will include a study of mechanistic overlaps between the actions of growth factors, oncogenes or oncogene products, and small effector molecules such as the retinoids on cells. One specialized aspect of TGF-beta is its ability to induce a local fibrotic response in vivo. Efforts will be focused on identifying the specific cell types involved in this response and studying their responses to TGF-beta in in vitro culture. An effort will be made to clarify the mechanistic basis for the bifunctional effects of both retinoids and TGFs on proliferation and transformation as well as for the observations that interactions between retinoids and TGFs are antagonistic in certain situations and synergistic in others.

Methods Employed:

Our laboratory has purified the type beta-TGF to homogeneity and is currently making milligram quantities of this peptide from human platelets. As a side-product of this purification, we hope also to make pure platelet-derived growth factor (PDGF) for use in our studies both in vitro and in vivo. For our studies with epidermal growth factor (EGF), we also purify this peptide to homogeneity from murine salivary glands. In collaboration with Genentech, Inc., we have specifically cloned cDNA probes for both types alpha and beta TGFs. Receptor assays for binding of TGF-beta to both cells and to solubilized membrane preparations have been developed. Radioimmunoassays for TGF-beta are currently being developed.

Methodology will include utilization of specific biochemical assays for growth factor production by cells, assays for proliferation of cells in monolayer culture and for growth of cells in semi-solid agar medium (used as a marker for in vitro transformation of fibroblastic cells), radioreceptor assays, radioimmunoassays, molecular hybridization including Northern and Southern blotting techniques, DNA transfection of cells in culture, light microscopy and scanning and transmission electron microscopy (in collaboration with Dr. U. I. Heine) and specific assays for cellular synthesis of matrix proteins such as collagen.

Major Findings:

There is now a solid base of data supporting a bifunctional role for TGF-beta in the regulation of cellular proliferation and phenotype. At one level, this bifunctionality can be demonstrated using different assay conditions; thus TGF-beta is inhibitory for growth of NRK cells in monolayer culture, but stimulatory for the growth of these same cells grown in soft agar under conditions of anchorage-independent growth. In this same cell line, effects of TGF-beta and EGF are antagonistic in monolayer culture, and synergistic in soft agar culture. If TGF-beta effects on the anchorage-independent growth of different cell lines are compared, it can be shown that while TGF-beta is stimulatory for growth of NRK cells, it is inhibitory for the growth of the majority of human tumor cell lines examined. The cell line most sensitive to inhibition by TGF-beta is a human lung carcinoma cell line, A549, in which the ED_{50} for inhibition is 0.4 pM TGF-beta. Finally, the bifunctional effects of TGF-beta can also be demonstrated in the same cell line under identical assay conditions, the effect of TGF-beta depending on the total set of growth factors presented to the cells together with TGF-beta. Thus the anchorage-independent growth of Fischer rat 3T3 cells transfected with a *myc* gene is inhibited by TGF-beta if EGF is also present, but stimulated by TGF-beta in the presence of PDGF.

Cell lines established in culture have undergone changes resulting in their immortalization. Some have likened these changes to preneoplasia. Thus the effects of TGF-beta on primary cultures of rat embryo fibroblasts were studied. PDGF had the greatest stimulatory effect on growth of cells in soft agar culture; TGF-beta was inhibitory for growth of the cells under both anchorage-dependent and anchorage-independent conditions. The ability of TGF-beta to antagonize the effects of PDGF suggested that secretion of TGF-beta by cells may, in certain instances, protect the cell from transformation.

Extension of previous studies on the effects of retinoids on growth factor-dependent proliferation of cells showed that retinoic acid has a selective effect on TGF-beta-dependent processes, suggesting that TGF-beta acts through cellular pathways distinct from those of mitogenic peptides such as EGF and PDGF. Thus in Fischer rat 3T3 cells transfected with a *myc* gene, retinoic acid inhibits the colony formation dependent on the combined effects of TGF-beta and PDGF but has no effect on the colony formation dependent on EGF alone. Additional experiments to determine whether the inhibitory effects of retinoic acid were directed at TGF-beta or PDGF showed that retinoic acid inhibits the colony growth of the cells whenever TGF-beta is growth stimulatory (as in the presence of PDGF) or growth inhibitory (as in the presence of EGF). These effects of retinoic acid and TGF-beta are specific for conditions of anchorage-independent growth of the cells. When cultured in monolayer, the growth of the cells is consistently inhibited by TGF-beta and unaffected or stimulated by retinoic acid. This suggests that the effects of these two factors are more closely related to transformation (as inferred from effects on anchorage-independent growth) than to growth *per se*.

Experiments directed at examining the levels of cellular expression of TGFs alpha and beta, using cloned cDNA probes for the peptides, suggest that in small cell lung cancer cells there may be a relationship between cellular growth patterns and TGF expression. Thus three cell lines that are able to grow under serum-free conditions have detectable levels of TGF-alpha expression, but not of TGF-beta. Conversely, in RNA preparations of cells that require serum for growth, TGF-beta message, but not TGF-alpha message, is detected. In one cell line, early passages of the cells that grow in suspension in serum-free medium have detectable transcription of TGF-alpha but not TGF-beta; later passages of the same cells that grow as attached monolayers have detectable transcription of TGF-beta but not of TGF-alpha. These correlations are being explored in greater depth.

In vivo experiments comparing the effects of TGF-alpha with those of EGF on the precocious eyelid opening of newborn mice have shown that the recombinant TGF-alpha (obtained from Genentech) is equipotent to EGF in this assay. This assay can now be added to the growing list of assays in which EGF and TGF-alpha, which has been shown to be essential for transformation of certain cells, behave identically. The results of the eyelid-opening assay are all the more remarkable considering that pharmacokinetic properties such as absorption and transport are also involved.

In vivo experiments designed to study the effects of TGF-beta on tissue repair have demonstrated that subcutaneous injection of TGF-beta into the nape of the necks of newborn mice results, in 2-3 days, in a typical granulation response with infiltration of inflammatory cells and proliferation of fibroblastic cells at the site of injection. This response was not produced by EGF used as a control. Increased collagen synthesis, as detected by staining of histologic sections, was also prevalent and was dependent upon the dose of TGF-beta administered. We are attempting to develop *in vitro* models for these effects using cultures of dermal fibroblasts.

Significance to Biomedical Research and the Program of the Institute:

It is clear from recent studies demonstrating the identity of PDGF and of the EGF receptor with known oncogene products, that growth factors and their receptors occupy a central position in carcinogenic transformation of cells as well as in certain other non-neoplastic proliferative states in which oncogene expression has been shown to be transiently increased, such as embryogenesis and tissue repair. Understanding of the mechanisms leading to enhanced synthesis of growth factors and their receptors by cells and of control of the effects of growth factors by low-molecular weight effectors such as the retinoids is fundamental to our understanding of both non-neoplastic and neoplastic proliferative states.

Proposed Course:

Future work will continue to focus on relationships between the mechanisms of action of growth factors, specifically type beta TGF, oncogenes, and retinoids, on cells. Specifically, we will attempt to transfect other cell lines with a *myc* gene to test the generality of the effects observed

with the transfected Fischer rat 3T3 cells. Other studies will center around further exploration into transcriptional controls on the genes for TGFs alpha and beta, with attempts to correlate changes in transcription with changes in behavior of cells. Attempts will also be made to develop *in vitro* models for the observed fibrotic response to TGF-beta *in vivo* by examining the ability of TGF-beta to induce synthesis of matrix proteins such as collagen in cultures of dermal fibroblasts. Effects of TGF-beta on lymphocytes will also be explored, since inflammatory cells are part of the natural response to tissue injury. A different *in vivo* model, the regenerating liver, will also be explored to possibly provide insight into the role of TGF-beta in tissue processes.

Publications:

Anzano, M. A., Roberts, A. B., De Larco, J. E., Wakefield, L. M., Assoian, R. K., Roche, N. S., Smith J. M., Lazarus, J. E., and Sporn, M. B.: Increased secretion of type beta transforming growth factor accompanies viral transformation of cells. *J. Molec. Cell. Biol.* 5: 242-247, 1985.

Derynck, R., Roberts, A. B., Winkler, M. E., Chen, E. Y., and Goeddel, D. V.: Human transforming growth factor-alpha: Precursor structure and expression in *E. coli*. *Cell* 38: 287-297, 1984.

Roberts, A. B., Anzano, M. A., Wakefield, L. M., Roche N. S., Stern, D. F., and Sporn, M. B.: Type beta transforming growth factor - a bifunctional regulator of cellular growth. *Proc. Natl. Acad. Sci. USA*, 82: 119-123, 1985.

Roberts, A. B., Roche, N. S., and Sporn, M. B.: Selective inhibition of the anchorage-independent growth of *myc*-transfected fibroblasts by retinoic acid. *Nature* 315: 237-239, 1985.

Smith, J. M., Sporn, M. B., and Roberts, A. B., Derynck, R., Winkler, M. E., and Gregory, H.: Human transforming growth factor-alpha causes precocious eyelid opening in newborn mice. *Nature* 315: 515-516, 1985.

Sporn, M. B. and Roberts, A. B.: Autocrine growth factors and cancer. *Nature* 313: 745-747, 1985.

Sporn, M. B., Roberts, A. B., and Driscoll, J. S.: Growth factors and differention. In Devita, V. T., Hellman, S., and Rosenberg, S. A. (Eds.): *Cancer: Principles and Practice of Oncology*. Philadelphia, J. B. Lippincott Company, Ed. 2, 1985, pp 49-65.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05267-04 LC

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Identification and Action of Platelet-derived Transforming Growth Factor-beta

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	Richard K. Assoian	Staff Fellow	LC	NCI
Others:	Barbara Fleurdelys	Bio. Lab. Tech.	LC	NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Chemoprevention

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
4.0	2.0	2.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Human platelets were extracted with acid-ethanol and platelet-derived TGF-beta was purified from the extract by a two-column procedure using sequential gel filtration in the absence and then presence of urea. Purified TGF-beta is a protein of 25,000-daltons, and it is comprised of two 12,500-dalton subunits held together by disulfide bonds. The purified factor elicits its biological activity at concentrations less than 4pM. Comparative studies showed that platelets contain 100 times more TGF-beta than do other non-neoplastic tissues. Platelets also contain a peptide growth factor related to EGF. These two new growth factors can interact mechanistically. Incubation of TGF-beta with NRK cells for 6 h results in an increased number of cell surface EGF receptors. IGF-II receptors are not affected. Shorter incubations with TGF-beta show that this peptide can also increase the Kd of the high affinity EGF receptor.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

Richard K. Assoian	Staff Fellow	LC	NCI
Barbara Fleurdelys	Bio. Lab. Tech.	LC	NCI

Objectives:

To examine the roles of bioactive peptides in modulating normal and neoplastic cell growth. Emphasis will be placed on 1) the isolation of transforming growth factors (TGFs) from platelets and 2) the mechanism by which platelet-derived TGF elicits a transformed phenotype.

Methods Employed:

Clinically outdated human platelets are extracted with acid-ethanol and the soluble peptides are precipitated with ether. The extract is purified by gel filtration, high pressure liquid chromatography and preparative gel electrophoresis. Biological activity is localized by use of an anchorage-independent growth assay with NRK fibroblasts. Peptides are chemically localized by polyacrylamide gel electrophoresis in conjunction with silver staining and analytical radioiodination. Purified TGF-beta is incubated with cultures of NRK cells. EGF receptors are measured with a radioreceptor assay. EGF action is measured on NRK cells by synergism with TGF-beta in soft agar or stimulation of ^{3}H-thymidine incorporation in monolayer.

Major Findings:

Platelets are the major non-neoplastic source of TGF-beta. The platelet-derived factor has been purified to homogeneity and shown to be a protein of 25,000 daltons comprised of two 12,500 dalton subunits. Disulfide bonds are involved in holding the subunits together. The primary structure of TGF-beta has been determined. Platelets also contain a peptide (M_r=27,000) which competes with labeled EGF for EGF receptor binding. TGF-beta in monolayer culture has a biphasic effect on EGF binding. It induces a transient decrease in receptor binding (due to a decrease in affinity) followed by a prolonged stimulation of binding (due to an increase in receptor number). Both of these effects have direct functional consequences for EGF-stimulated mitosis.

Significance to Biomedical Research and the Program of the Institute:

The presence of a transforming growth factor in platelets (a non-neoplastic cell fragment) indicates that these peptides have important roles in the control of normal cell growth. The mechanistic interaction of these TGF-beta and the EGF-like peptides (via control of EGF receptors) provides a novel mechanism for analyzing the biological controls on cell division.

Proposed Course:

The mechanism of action and biochemical effects of platelet-derived TGF-beta will be examined with cell cultures of NRK fibroblasts and smooth muscle. Cytoskeletal proteins and extracellular matrix proteins will be examined for alterations in response to TGF-beta.

Publications:

Assoian, R. K.: Biphasic effects of type beta transforming growth factor on epidermal growth factor receptors in NRK fibroblasts: functional consequences for epidermal growth factor-stimulated mitosis. J. Biol. Chem. (in press).

Assoian, R. K., Grotendorst, G. R., Miller, D. M. and Sporn, M. B.: Three peptide growth factors from human platelets coordinating phenotypic transformation. Nature 309: 804-806, 1984.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05396-02 LC

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT (*80 characters or less. Title must fit on one line between the borders.*)

Development of Analogs for Study of Oncogenesis and Development of the Rat

PRINCIPAL INVESTIGATOR (*List other professional personnel below the Principal Investigator.*) (*Name, title, laboratory, and institute affiliation*)

PI:	Shinichi Watanabe	Senior Staff Fellow	LC	NCI
Other:	Eliane M. Lazar	Visiting Fellow	LC	NCI
	Myung Kim	Visiting Fellow	LC	NCI
	Kondaiah Paturu	Visiting Fellow	LC	NCI
	Linda L. Dart	Biologist	LC	NCI
	Gloria M. Sundaresan	Chemist	LC	NCI

COOPERATING UNITS (*if any*)

None

LAB/BRANCH

Laboratory of Chemoprevention

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.7	1.6	1.1

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK (*Use standard unreduced type. Do not exceed the space provided.*)

Several mutations have been introduced into a cloned human TGF-alpha gene by site-directed mutagenesis. These mutants will be expressed to study structure-activity relationships of TGF-alpha. The rat TGF-alpha gene has been chemically synthesized for further in vivo studies in rats. The rat TGF-alpha gene will be inserted into a vaccinia virus vector and then used to immunize rats against TGF-alpha activity. An anti-sense rat TGF-alpha gene has been constructed using a retrovirus vector and will be used in an attempt to inhibit specific expression of TGF-alpha.

PHS 6040 (Rev. 1/84)

PROJECT DESCRIPTION

Names, Title, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

Shinichi Watanabe	Senior Staff Fellow	LC	NCI
Eliane M. Lazar	Visiting Fellow	LC	NCI
Myung Kim	Visiting Fellow	LC	NCI
Paturu Kondaiah	Visiting Fellow	LC	NCI
Linda L. Dart	Biologist	LC	NCI
Gloria M. Sundaresan	Chemist	LC	NCI

Objectives:

We are studying the role of TGF-alpha in normal and malignant cells and are attempting to convert transformed cells into normal cells by producing antagonists against the transforming factor TGF-alpha.

Methods Employed:

Human TGF-alpha gene was mutated by oligonucleotide directed mutagenesis in the M13 vector. The modified genes were transferred to yeast vector to express properly folded TGF-alpha. The rat TGF-alpha gene was chemically synthesized by using the automated DNA synthesizer. This gene was inserted into a retrovirus vector to produce rat TGF-alpha in animal cells. The retrovirus vector was used to construct anti-sense clones of rat TGF-alpha, *ras*, and *sis* genes.

Major Finding:

We have synthesized the complete rat TGF-alpha gene from a published amino acid sequence. We have also introduced mutations in the human TGF-alpha gene: deletions at amino acid positions 38, both 37 and 38, 40, and 42, and disruptions of disulfide bends at positions 8-21, 16-32 and 34-43.

Significance to Biomedical Research and the Program of the Institute:

Although the levels of TGF-alpha and TGF-beta are elevated in cells transformed by various carcinogens, the role of these growth factors in carcinogenesis is not known. The development of analogs for TGF-alpha will be very useful tools to examine the role of the growth factor during carcinogenesis. The analogs will be useful for understanding the growth factor-receptor interaction at the molecular level. The role of TGF-alpha during developmental processes of an animal is not known. Embryo transplantation after introduction of altered genes will provide valuable information. The overall information obtained with analogs of TGF-alpha and TGF-beta will help to develop practical methods to reverse/prevent transformation.

Proposed Course:

The mutated human TGF-alpha genes will be expressed in a yeast system. After purification of each mutated TGF-alpha, we will test its biological activity (formation of colonies in soft-agar, receptor binding competition with EGF). Anti-sense clones constructed in a retrovirus expression vector will be used to transfect cells and to determine if each gene is essential for the functions. A vaccinia virus vector will be used in an attempt to immunize animals against excessive TGF-alpha.

Publications:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05397-02 LC

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Role of Ectopic Peptides in Phenotypic Transformation

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	Joseph E. De Larco	Research Chemist	LC	NCI
Others:	Dennis Pigott	Visiting Associate	LC	NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Chemoprevention

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.0	2.0	0.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

A cell line was derived from a metastatic, human melanoma. It is producing peptides that can stimulate an untransformed cell line to reversibly express a transformed phenotype. This transformed phenotype is expressed in monolayers of untransformed cells as a disorganized growth pattern and in anchorage-independent growth (AIG) assays as colonies forming in soft-agar from single seeded cells. One of the peptides in this mixture contributing to this activity is an epidermal growth factor-like growth factor that has a molecular weight of approximately 24,000 daltons as determined by gel permeation chromatography on Bio-Gel P-30 in 1 M acetic acid. This appears to be present at high concen trations (2.5 g/liter). The mitogenic activity present in these fractions coelutes with the EGF-like activity. Two additional activities are eluted from the Bio-Gel column. The first of these coelutes with the EGF-like activity and appears to mitigate cellcell interactions, both in the cells of induced agar colonies and in monolayers of NRK cells. The last activity eluting from the column is a TGF-beta. This TGF-beta activity has an apparent molecular weight of approximately 14,500 daltons. These "ectopic" peptides may play a role in the expression of the transformed phenotype of the tumor cells producing them.

Molecular clones have been derived from sequences regulated by the combination of EGF and TGF-beta. The identification of these induced sequences will, hopefully, add to understanding of the role of these factors in the expression of the transformed state.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliation of Professional Personnel Engaged on this Project:

Joseph E. De Larco	Research Chemist	LC	NCI
Dennis Pigott	Visiting Associate	LC	NCI

Objectives:

One of the primary objectives is the production of immunological reagents against the epidermal growth factor-like growth factors and the modulators released by a human melanoma cell. Taking advantage of a melanoma cell line that produces large quantities of an apparently high molecular weight EGF-like growth factor that may be analogous to that released in the urine of cancer patients, we will isolate this material from serum-free media conditioned by these melanoma cells *in vitro*. Enough of this material will be isolated for amino acid sequence determination and the production of immunological reagents. Knowing the amino acid sequence, antisera can be generated against peptide sequences that are specific to this particular EGF-like growth factor. With specific reagents it will be possible to determine if these large molecular weight growth factors are more closely related to human EGF (urogastrone), the transforming growth factors (TGFs) or a yet undescribed family of EGF-like peptides. The possibility of using these reagents as diagnostic tools to determine if there are any qualitative or quantitative differences in the EGF-like peptides expressed in patients having certain malignancies will be examined using urine samples from a variety of patients. The possibility of using these antibodies as therapeutic agents will also be explored. Specific, high titered antibodies against these factors will allow one to examine for the expression of these factors under normal development and physiological conditions. These reagents, along with cell culture systems, will be used to determine if there is a correlation between the expression of these genes, or closely related genes, and the transformed phenotype.

Along similar lines the cellular genes that are regulated by these growth and modulation factors will be isolated using molecular cloning techniques. These isolated clones will be used to study the expression of the transformed phenotype both *in vitro* and *in vivo*. They will also be used to study the developmental processes and basic mechanisms for growth control.

Cell clones have been isolated to determine what genes must be expressed for the indicator cells to manifest the transformed phenotype when treated with the growth and modulatory factors released by transformed cells.

Methods Employed:

Tissue culture methods are used for both the production of growth and modulatory factors as well as for assaying these activities. The tissue culture methods include roller bottles for isolation of both serum-free conditioned media and mRNAs. Mitogenic and soft agar assays are used to quantitate and characterize the ectopic factors released by transformed cells. Standard biochemical methods are used to isolate the factors released by the transformed cells. These include centrifugation, high pressure liquid chromatography column chromatography, gel exclusion chromatography, ion exchange chromatography and polyacrylamide gel electrophoresis. Molecular biological techniques are used to clone the regulated genes. These methods include the purification of poly(A) containing mRNA, the cloning of the poly(A) containing RNA and the characterization of the individual clones using the appropriate probes.

Major Findings:

Melanoma cells: Single cell clones were picked from a human melanoma cell line that had been established from a surgically isolated metastatic tumor. The individual clones differed from one another with respect to their growth properties and the amount of "ectopic" peptide growth factors they released. The major mitogenic activity released by these cells is an EGF-like peptide. Other peptide factors are released by these cells. They have modulatory activities but are not mitogenic in our assays. One of these modulating activities elutes with the EGF-like activity; this activity appears to decrease the cell-cell interactions in either the induced agar colonies or cells established in monolayers. The other modulating activity elutes from the Bio-Cel P-30 column with an apparent molecular weight of approximately 15K daltons. The production of the ectopic peptides or TGFs appear to be correlated with the growth properties and phenotypes expressed by the individual transformed clones producing these factors. The clones that grew better in agar also released more growth and modulating factors. Selected clones are producing much more growth factors than previously reported human melanoma lines. In the literature it has been reported that human melanoma cells release between 10 and 20 nanograms of an EGF-like peptide per liter of conditioned media. These clones, however, release between 1,000 and 2,500 nanograms of a EGF-like peptide per liter of conditioned media. The EGF-like activity from these cells has an apparently larger molecular weight than had previously been reported for melanoma cells. Its apparent molecular weight is approximately 24,000 compared to 7,000 for the TGF-alpha reported for other melanoma lines. This molecular weight appears to be analogous to the peptides found in the urine samples from cancer patients that have certain types of tumors.

This could be a very useful cell line for the production of immunological reagents to be used as diagnostics in the early detection of malignant tumors. It can supply antigens for the production of antibodies for screening urines of potential cancer patients for the presence of these or similar peptides. Antibodies to these peptide growth factors released by the melanoma cells may be very useful as diagnostic tools for the detection of certain malignancies. This melanoma line also will be used to study the biochemistry and the cellular biology of the transformed cells producing these factors.

These cells produce other modulating factors that have not been characterized as yet. These modulating activities appear to contribute to the transformed phenotype. This can be seen in the indicator cells when they are treated with serum-free conditioned media from these melanoma cells. These modulators are being examined presently for their ability to influence the metastatic potential of transformed cells. An understanding of the mechanism of action of these factors and/or the ability to control their expression would be very useful in the treatment of malignant melanomas. The ability to prevent the migration of tumor cells from the primary tumor would, hopefully, minimize the metastatic potential of tumors in vivo.

Proximal Effectors of the Transformed Phenotype: It has been observed that when serum-free conditioned media from either murine sarcoma virus-transformed cells or human melanoma cells is added to the media in which untransformed indicator cells (49F) are growing, they express the transformed phenotype. This phenotype is expressed if the cells are treated with inhibitors of DNA synthesis. The transformed phenotype, however, is not induced if the cells being treated with the serum-free conditioned media are treated with inhibitors of either RNA or protein synthesis. This suggests that the cells must synthesize mRNA and protein before expressing the transformed phenotype. Similar phenotypic transformation is obtained if the 49F cells are treated with EGF and purified TGF-beta from platelets. It is assumed that the combination of these two factors turns on the expression of a "silent" gene that is not normally expressed by these cells. These "silent genes" may be required for the expression of the transformed phenotype. This property would make the product(s) from these genes more proximal effectors of the transformed phenotype or transformation. The identity of these "proximal effectors of transformation" would therefore be of great interest in understanding transformation as well as understanding the regulatory mechanisms for growth and differentiation. To accomplish this a series of these "silent genes" have been cloned from the mRNAs of cells that have been stimulated. These cDNA clones will be used to try to dissect out the steps in transformation. At present a series of cellular clones of the original indicator cells, 49F, have been isolated for this purpose. The cDNA clones of the "proximal effectors" will also be used to examine transformed and untransformed cells from in vitro and in vivo sources. These clones will be helpful in establishing the expression of the regulated genes during development and the progression of the transformed phenotype.

Significance to Biomedical Research and the Program of the Institute:

This work is both significant and of high priority because the results from these experiments will continue to provide information that will hopefully help in defining the factors responsible for the uncontrolled proliferation and altered social behavior expressed by malignant cells. The data collected from the human melanoma system would appear to be directly applicable for the development of diagonstics for human malignancies. The in vitro system developed will allow the rational evaluation of methods for early detection and screening of possible therapeutic agents for the treatment of these malignancies. Two classes of therapeutic agents can readily be screened using these systems. The first consists of those that suppress the production or release of the factors responsible for the expression of the transformed

phenotype. The second includes those agents that decrease the response of the tumor cells to the "ectopic" factors. These goals are consistent with those of the Laboratory of Chemoprevention, the Division and the NCI. It is hoped the information gained also will add to our basic knowledge of growth control and differentiation. This knowledge will provide methods and rationale that can be applied to patient care for both early diagnosis and treatment.

Proposed Course:

This project represents an integrated approach to understanding the roles of the "ectopic" growth and modulatory factors in the expression of the transformed phenotype. Future studies are to proceed in a logical manner using the basic knowledge already accumulated. This affords the opportunity to prepare immunological and molecular biological reagents to study the control mechanisms for the expression of these factors during normal growth and differentiation, as well as during malignant transformation. Immunological reagents developed will be used to monitor the expression of the "ectopic" growth and modulatory factors by malignant tumors in cancer patients before and during treatment. With the cellular clones isolated the cellular and molecular biology of the expression, production and release of EGF and EGF-like peptides will be examined. The development of molecular probes for these peptides should afford insight into the normal expression and function of these potent mitogens.

Publications:

De Larco, J. E., Pigott, D. A., and Lazarus, J. A.: Ectopic peptides released by a human melanoma cell line that modulate the transformed phenotype. Proc. Natl. Acad. Sci. USA (in press).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05398-02 LC

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Functional Characterization of Transforming Growth Factors and Their Receptors

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	Lalage M. Wakefield	Visiting Fellow	LC	NCI
Other:	Bradford O. Fanger	Guest Researcher	LC	NCI
	Diane M. Smith	Biologist	LC	NCI

COOPERATING UNITS (if any)

Genentech, Inc., South San Francisco, California

LAB/BRANCH

Laboratory of Chemoprevention

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.8	2.0	0.8

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Transforming growth factors (TGFs) are acid-stable polypeptides that induce a reversible phenotypic transformation of normal indicator cells such that they will grow in an anchorage-independent manner in soft agar, a property characteristic of transformed cells. The purpose of this project is to determine the role that endogenously-produced TGFs may play in the growth of normal and transformed cells and to characterize their mode of action at a biochemical level. To this end, polyclonal antisera have been raised to the TGFs and development of monoclonal antibodies is in progress. The effects of these antibodies on the anchorage-dependent and -independent growth of normal and transformed cells are being investigated and will be analyzed in terms of the biochemical functions affected. Since the first step in the interaction of TGFs with the cell is binding of the growth factor to the cell surface, initial investigations have concentrated on the characterization of cell surface receptors for TGFs. Development of radioreceptor assay for TGF-beta, a TGF that depends on a second TGF (EGF or TGF-alpha) for activity, has allowed identification of a specific high affinity receptor for TGF-beta on all normal and transformed cell lines studied so far. The receptor appears to be a disulphide-linked dimer that does not undergo ligand-induced autophosphorylation or clustering. Receptor properties are modulated by transformation but binding of TGF-beta to its receptor does not appear to be a major control point in TGF-beta action. Human cell lines and tissues are being screened for an abundant source of the receptor for use as a starting material for receptor purification and as an immunogen for the production of anti-receptor monoclonal antibodies. Further characterization of the role of endogenously-produced TGFs and their interaction with the cell surface receptor should help elucidate the role these molecules may play in the process of carcinogenesis.

PROJECT DESCRIPTION

Names, Title, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project

Lalage M. Wakefield	Visiting Fellow	LC	NCI
Bradford O. Fanger	Guest Researcher	LC	NCI
Diane M. Smith	Biologist	LC	NCI

Objectives:

The purpose of this project is to examine the role of transforming growth factors (TGF) in the control of normal cell growth and in the process of malignant transformation. Anti-TGF antibodies are being employed to investigate the involvement of endogenously-produced TGFs in growth control in normal and transformed cells. The mechanism of action of these growth factors is being studied at a biochemical level with particular emphasis on the initial interaction of the TGFs with the cell surface receptor; the first step is the cascade of events leading to expression of the transformed phenotype. With a greater understanding of the importance and mechanism of action of TGFs in transformation, analogs and potential inhibitors may be synthesized and tested with a view to producing effective chemotherapeutic agents.

Methods Employed:

A method has been developed for the radioiodination of TGF-beta to high specific activity with essentially no loss of biological activity. This iodinated material is used in a radioreceptor assay to characterize TGF-beta binding to a variety of cell lines in culture and binding data are subjected to Scatchard analysis. Biochemical characterization of the receptor has involved chemical cross-linking of radiolabeled ligand to the receptor followed by analysis of cross-linked species by SDS polyacrylamide gel electrophoresis and autoradiography. A soluble receptor binding assay has also been developed. Polyclonal antisera to TGF-beta are being raised in rabbits and goats and monoclonal antibodies against this growth factor and TGF-alpha are being developed in collaboration with Genentech, Inc. Immunoglobin fractions from the antisera are prepared by affinity chromatography and antibody titer is determined by Elisa and radioimmunoassays. The ability of cells to grow in an anchorage-independent manner in soft agar is used as an assay for the transformed phenotype and the number and size of cell colonies obtained is measured using an Omnicon image analyzer. Anchorage-dependent growth is quantitated by determining changes in cell number for cells grown in monolayer.

Major Findings:

Characterization of the TGF-beta Receptor

Binding of TGFs to the cell membrane initiates the chain of events leading to cell transformation, so TGF-receptor interaction and its modulation are of particular interest in any mechanistic studies. A method was developed for the iodination of TGF-beta without loss of biological activity for use in a radio-

receptor assay. The normal rat kidney (NRK) cell line which responds to TGF-beta (in the presence of epidermal growth factor or TGF-alpha by growth in soft agar) was shown to possess a specific high affinity receptor for TGF-beta.

There are 25,000 such receptors per cell, with a Kd of 40 pM. Other growth factors such as platelet-derived growth factor, EGF, TGF-alpha insulin and insulin-like growth factors I and II do not compete for binding to this receptor, so unlike TGF-alpha which binds to the previously characterized EGF receptor, TGF-beta appears to bind to a distinct receptor. The existence of a single class of binding site indicates that both the positive and negative growth modulatory effects of TGF-beta must be exerted through the same receptor. Binding of TGF-beta is a time and temperature-dependent process and at 37° the bound ligand is rapidly internalized and degraded in the lysosomes. TGF-beta will down-regulate its receptor to a maximum of 30-50% of the levels initially observed. Similar high affinity receptors have been found on all cell lines assayed so far with the rodent cell lines, NIH-3T3 and Swiss 3T3, expressing the highest number of receptors, at 60,000-90,000 per cell. The human cell lines studied so far have many fewer receptors (less than 10,000 per cell) and there is no obvious correlation between the number or affinity of the receptor and the tissue source of the cell line (normal or tumor, adult or embryonic) or the ability of the cell to grow in soft agar.

Modulation of receptor expression

Transformation of rodent cell lines by acute transforming retroviruses was shown to cause a marked decrease in the number of TGF-beta receptors. Transformation of NRK cells by MoSV and of NIH-3T3 cells by HaSV results in a 50% decrease in TGF-beta binding. This decrease in receptor number correlates with increased expression of TGF-beta by the transformed cells and is probably due to receptor down-regulation or occupation by endogenous TGF-beta. A similar effect has been observed in virally transformed cells that secrete TGF-alpha or PDGF, where there is an accompanying loss of assayable cell surface receptors for these ligands. Similarly, transfection of Fischer rat 3T3 cells with the oncogenes *myc* and *ras* cause a decrease in TGF-beta receptor number. Conversely, however, transformation by the DNA virus SV40, or chemical or spontaneous transformation result in increased numbers of receptors for TGF-beta. The significance of this modulation in receptor properties on cellular transformation is being investigated.

Biochemical Characterization of TGF-beta Receptor

Chemical cross-linking studies have shown the TGF-beta receptor to be a disulphide-linked dimer with a molecular weight of approximately 360 Kd. Unlike most other growth factor receptors, the TGF-beta receptor does not appear to undergo ligand-induced clustering or phosphorylation. A soluble receptor binding assay has been developed which will aid in the purification and further characterization of the receptor and associated enzyme activities.

Antibodies to TGF-beta

Polyclonal antibodies have been raised against human platelet TGF-beta. Immunoglobulin fractions have been prepared from this serum by affinity chromatography

on protein A-sepharose. These antibodies effectively block binding of iodinated TGF-beta to its receptor and inhibit the transforming effect of exogenously-added TGF-beta on NRK cells (in combination with EGF) as measured by the soft agar growth assay. These antibodies are now being studied for an effect on anchorage-dependent and -independent growth of cells that synthesize TGF-beta to determine the role of endogenous TGF-beta in growth control, and they have been used to show that TGF-beta is the primary differentiation-inducing agent for normal bronchial epithelial cells in whole blood serum. Similarly a monoclonal antibody to TGF-alpha has been shown to block the growth of a human rhabdomyosarcoma line *in vitro*. This work is currently being extended to other human tumor lines.

Significance to Biomedical Research and the Program of the Institute:

Characterization of the roles of TGFs in initiating or maintaining the transformed phenotype, and their mechanism of action and relationship to normal growth factors in both normal and tumor cells, will give an insight into cellular growth control processes that may provide a rational basis for the development of effective chemotherapeutic agents for the inhibition of carcinogenesis.

Proposed Course:

Future research will continue on two main areas. The first involves the continued use of anti-TGF antibodies to determine what role endogenously-produced TGFs play in controlling growth and differentiation of normal and transformed cells. It is anticipated that the various TGFs may have different regulatory functions in different cell types. Since the first step in the mechanism of TGF action is their interaction with the cell surface receptor, the other major research interest will be to characterize these receptors further. Particular emphasis will be placed on raising monoclonal antibodies against the TGF-beta receptor for use in an affinity column for the purification of the receptor.

Publications:

Anzano, M. A., Roberts, A. B., De Larco, J. E., Wakefield, L. M., Assoian, R. K., Roche, N. S., Smith, J. M., Lazarus, J. E., and Sporn, M. B.: Increased secretion of type beta transforming growth factor accompanies viral transformation of cells. J. Mol. Cell. Biol. 5: 242-247, 1985.

Frolik, C. A., Wakefield, L. M., Smith, D. M. and Sporn, M. B.: Characterization of a membrane receptor for transforming growth factor-beta in normal rat kidney fibroblasts. J. Biol. Chem. 259: 10995-11000, 1984.

Roberts, A. B., Anzano, M. A., Wakefield, L. M., Roche, N. S., Stern, D. F., and Sporn, M. B.: Type beta transforming growth factor: a bifunctional regulator of cell growth. Proc. Natl. Acad. Sci. USA 82: 119-123, 1985.

ANNUAL REPORT OF

THE LABORATORY OF COMPARATIVE CARCINOGENESIS

NATIONAL CANCER INSTITUTE

October 1, 1984 through September 30, 1985

The Laboratory of Comparative Carcinogenesis (LCC) plans, develops and implements a research program in experimental carcinogenesis. The Laboratory (1) compares effects of chemical carcinogens in rodents and nonhuman primates to identify determinants of susceptibility and of resistance to carcinogenesis; (2) identifies, describes, and investigates mechanisms of interspecies differences and of cell and organ specificities in carcinogenesis; (3) investigates the roles of nutrition, metabolism, the perinatal age period and pregnancy in modifying susceptibility to chemical carcinogens; and (4) conducts biological and morphologic studies on the pathogenesis of naturally occurring and induced tumors in experimental animals.

Summary Report: The Laboratory of Comparative Carcinogenesis provides a major focus within the Chemical and Physical Carcinogenesis Program for studies on the mechanisms of chemical carcinogenesis that involve primary neoplasia in animals as experimental endpoints. An increasing volume of evidence continues to support the hypothesis that for many, if not most tissues, transient exposure to chemical carcinogens either before or after birth may be necessary but is not sufficient to elicit tumor development. The widely differing patterns of organ specificity that frequently occur in experimental carcinogenesis in different species, even in studies with direct-acting agents that are independent of cellular metabolism, are in many cases not explicable on the basis of toxicodynamics, nor on the basis of differential capacity to repair damage in different tissues. These findings complicate efforts at human risk assessment based on the extent of reaction or persistence of binding products of carcinogens in known animal and putative human target tissues. In addition, more and more agents are being identified that cause tumors in experimental rodents but do not react chemically with cellular constituents including DNA, i.e., are not genotoxic. The fact that potential tumor cells may remain latent for large fractions of a lifetime in experimental animals, and that increasing numbers of nongenotoxic agents are being discovered which promote proliferation of such latent cells to form preneoplastic lesions that progress to neoplasia suggest that the phenomenon of tumor promotion may be of great significance for the genesis of human cancer.

There is, at present, no unifying hypothesis for the general mechanism of action of tumor promoters other than the concept that such agents reduce intercellular communication. Furthermore, most experimental studies on tumor promotion in specific tissues or organ systems have focused on one, or at most, two species, and the empirical data base from which mechanistic hypotheses of tumor promotion will eventually emerge remains very narrow. Accordingly, there is no certainty at present that agents identified as promoters in rodent tissues will have similar effects in other species, including man. In order to expand the limited data base on organ specificity and interspecies correlations in tumor promotion, a major coordinated program has been established in this Laboratory to identify previously unsuspected promoting agents; to establish rigorously the limits of cellular specificity for tumor promotion by specific agents; to compare dose/effect relationships from one species to another, including both rodent and nonhuman primate species; and to

utilize these data in mechanistic investigations on the phenomena of tumor promotion. Identification of specific transforming genes (oncogenes) in both human and experimental tumors has stimulated great interest and intensive efforts in many laboratories to clarify the roles of those genes in the pathogenesis of cancer. As many of the known oncogenes derive from normal elements of the mammalian genome (Hunter, T., JNCI 73: 773-785, 1984), there is a real possibility that the biochemical mechanisms of neoplastic transformation may be definable by thorough analysis of the properties of the oncogenes and their cellular homologs. Of special interest in chemical carcinogenesis are oncogenes that behave as dominant genetic elements and that are activated to this behavior either by single-base transition mutation, such as the ras family of genes (Sukumar, S. et al., in Genes and Cancer, Liss, New York, 1984, pp. 353-371) or by chromosomal rearrangement, as in the case of myc (Alitalo, K., et al., PNAS 80: 1701-1711, 1983). Both of these mechanisms of activation can be caused in principle by genotoxic chemical carcinogens, which can be provisionally identified as such by their mutagenic or clastogenic effects. The mechanisms of action of such agents, long considered to involve damage to DNA, may eventually be reconciled with molecular virology, and the crucial events in cellular transformation by both chemical and biological agents defined, through analysis of the activation and behavior of oncogenes. Detection and critical evaluation of the roles of dominant oncogenes, especially mutant genes of the ras family, is also a major unifying theme in the research activities of several Sections within this Laboratory.

Major research programs on metabolic determinants of transplacental carcinogenesis in rodents and nonhuman primates, on carcinogenesis by salts of heavy metals, on the chemistry and biochemistry of nitrosamines, and on the role of nutritional deficiencies in chemical carcinogenesis also continue and are described in detail both in the following summary reports of each Section and in the individual project reports.

In addition to laboratory research, some individuals within LCC have been instrumental in organizing symposia and in editing the proceedings for publication. Proceedings of the Second World Congress on Trophoblast Neoplasms (Singapore, November 1984) and the Symposium on the Role of Essential Nutrients in Carcinogenesis (Bethesda, January 1985) are being edited for publication as NCI Monographs by Jerry Rice and Lionel Poirier, respectively. Finally, Larry Keefer organized an international conference on Organic and Biological Chemistry of Carcinogenic and Carcinostatic Agents Containing Nitrogen-Nitrogen Bonds, held at Harpers Ferry, WV, in May 1985.

The Office of the Chief (1) organizes comparative research on mechanisms of chemical carcinogenesis in susceptible and resistant species of experimental animals, (2) arranges and fosters collaborative approaches to specific research projects involving several Sections within and independent investigators outside the Laboratory, and (3) provides general support and direction to the intramural research program of the Laboratory.

In the Developmental Biology Working Group, four models of chemically induced tumors in F344 rats are currently being studied for the appearance and expression of activated oncogenes: renal mesenchymal tumors induced by methyl(methoxymethyl)nitrosamine (DMN-OMe), intestinal adenocarcinomas induced by methyl (acetoxymethyl)nitrosamine (DMN-OAc), neurogenic tumors transplacentally induced by nitrosoethylurea (NEU), and hepatocellular carcinomas induced by intraportal injection of DMN-OAc followed by phenobarbital promotion. Rats exposed neonatally to DMN-OMe developed predominantly nonmetastatic but highly proliferative renal mesenchymal tumors that were either sarcomatous or blastemal in appearance; however, one nasal cavity

adenocarcinoma with a high mitotic index was also observed. In addition, livers from most animals contained preneoplastic lesions and occasionally hepatocellular tumors. DNA preparations from 13 of 27 renal tumors and the 1 nasal cavity carcinoma were able to produce morphologic transformation in NIH/3T3 mouse cells. Similar preparations from the liver, brain, or contralateral nontumorous kidney of treated animals were negative in transfection experiments. The presence of rat repetitive sequences in the transformants was consistent with a postulated role of rat genes in the transformation of NIH/3T3 cells. Southern blot analysis of transformants derived from the renal and nasal cavity tumor DNA preparations revealed the presence of DNA fragments specific to the rat Ki-ras locus. Activation of Ki-ras appears to be more than a simple amplification of mRNA expression since transfection-positive renal tumor cells expressed levels of Ki-ras mRNA sequences similar to those of normal adult rat kidney by quick blot analysis. Preliminary studies to identify a specific point mutation in the Ki-ras gene at the 12th codon using synthetic oligonucleotide probes have not yet been successful. At the temperature tested, nonadecamers containing alterations of G to A or G to T in the second position of the Ki-ras 12th codon did not hybridize selectively with Hind III restriction fragments of DNA from transformants. Evaluation of tumor tissues from the three other tumor models is currently in progress.

The consistent demonstration of a specific dominant transforming gene, Ki-ras, in F344 rat renal mesenchymal tumors induced by a single exposure to an alkylating agent adds another category of chemically induced neoplasm to the list of those that are known to be oncogene-associated, and because its histogenesis is well defined it should be possible to study the temporal sequence of expression of Ki-ras in relation to development of the neoplasm. As the result of the F344's use in standard bioassays for carcinogenic potential, and its common use in experimental oncology studies, abundant literature is available on the induction of neoplasms by a wide variety of chemical agents. Since the effects of chemicals in the animal model are inferred to predict human risk and act by mechanisms common to other species, the model system should reveal mechanisms relevant to human carcinogenesis.

The Perinatal Carcinogenesis Section investigates the induction of cancer in experimental animals before birth and during infancy; evaluates perinatal exposures to chemical carcinogens, inducers of xenobiotic metabolism, and tumor promoters as causative factors in pediatric and adult forms of human cancer; studies the effects of exposure to carcinogens during pregnancy; and investigates the relation of cellular differentiation to perinatal susceptibility to chemical carcinogens and to the consequent development of neoplasia.

The Section has been reduced in size and the scope of its research program narrowed by the recent transfer of some of its projects and staff to other units. The study of transplacental carcinogenesis in nonhuman primates is now carried out by a separate Primate Research Working Group in the Office of the Chief; projects relating to renal differentiation and expression of the neoplastic phenotype continue in the Developmental Biology Working Group; and systemic promotion of transplacentally initiated neoplasia by postnatal exposure to barbiturates and related compounds continues to be studied in the Tumor Pathology and Pathogenesis Section. These projects are described in the reports of the other Section and Working Groups, with which active collaboration continues. Study of organ-specific patterns of DNA repair capacity during development has essentially ceased with the departure of Dr. Beatrice Chen. The current program of the Section is focused on four projects directed by Dr. Lucy Anderson.

A major goal of the Perinatal Carcinogenesis Section is the identification and mechanistic study of the factors that affect and modulate the causation and appearance of tumors originating from transformed cells in the fetus or neonate. These factors may include maternal processing of carcinogens, interactions of these chemicals with the immature organs, and influences on the development of the initiated neoplasms. Some of these factors pertain to the adult animal as well, and in some cases it has seemed advantageous to turn, after a germinal discovery in a perinatal system, to the adult, which is more amenable to experimentation, in order to obtain clarification. With the new insight gained, one may then again address the more challenging fetal system. Thus, two of the four projects presented here include only experiments with adult animals, although both were inspired by the outcome of transplacental carcinogenesis experiments. These four projects, though diverse in approach, have a common thread--the search for mechanisms governing sensitivity to chemical carcinogenesis, particularly in the perinatal animal.

Studies on the protective role of induction of carcinogen-metabolizing enzymes in tumorigenesis originated from the observation that pretreatment of pregnant C57BL/6 mice with the noncarcinogenic inducer of mixed function oxygenase (MFO) enzymes, beta-naphthoflavone (beta-NF), effected partial protection of their fetuses from lung tumorigenesis by 3-methylcholanthrene (MC) (Anderson, L.M. and Priest, L.J., Res. Comm. Cm. Pathol. Pharmacol. 30: 431-446, 1980). A search of the literature, especially reviews by Wattenberg (e.g., JNCI 60: 11-18, 1978) revealed that pretreatment with enzyme inducers almost invariably results in reduction of a carcinogen's effect. This principle seems remarkable in its lack of exceptions and promising in its potential for human application, but has been rather overlooked, possibly because of preoccupation with the activation-to-proximate-forms aspect of metabolism. A systematic evaluation of the effects of enzyme induction on tumorigenesis in mouse strains of varying responsiveness to inducers seems not to have been reported. We therefore undertook such a study, with beta-NF as the inducer, intragastric MC as the carcinogen, and mice of six different genetic strains as subjects. The results have provided strong confirmation of the protective potential of enzyme induction.

These studies were extended to evaluation of roles of fetal and maternal metabolism of carcinogens in transplacental carcinogenesis, and to investigation of whether maternal and fetal genotypes governing responsiveness to induction of MFO enzymes play a determining role in sensitivity to transplacental carcinogenesis. In a now-classical pharmacogenetic model system developed by Nebert and colleagues, involving genetic crosses of C57BL/6 (induction responsive) and DBA/2 (nonresponsive) mice, sensitivity to tumorigenesis by subcutaneous MC, and to toxic effects by polycyclic aromatic hydrocarbons in several systems, has been found to correlate positively with responsiveness to induction. The importance of such responsiveness in fetuses has never been successfully addressed experimentally prior to our undertaking, although ontogenetic appearance of activating enzymes is often postulated to be a limiting factor in fetal susceptibility to tumorigenesis. We therefore carried out a transplacental dose-response assessment with MC in the C57/DBA model, using both responsive (C57BL/6 x DBA/2)F_1 and nonresponsive DBA/2 mothers. Two clear results were obtained. Fetuses which were responsive to induction of aryl hydrocarbon metabolism developed 2-3-fold more lung and liver tumors than did nonresponsive littermates, at almost every MC dose, confirming that this characteristic is indeed an important determinant of fetal susceptibility. Secondly, fetuses of nonresponsive mothers developed many more tumors after the same dose of MC than did fetuses of responsive dams; the fetus, like other distal target organs, is probably protected by enzyme induction in the mother.

Studies on the long-term effects of polychlorinated biphenyls (PCBs) on tumor development in mice was undertaken as a result of the unexpected observation of an apparent promotive effect of Aroclor 1254, a mixture of PCBs, on liver tumors initiated by dimethylnitrosamine (DMN) in infant mice, even though the PCBs were administered to the pregnant mother and were received only during the suckling period (Anderson et al., JNCI 71: 157-163, 1983). Aroclor 1254 and other commercial PCB mixtures contain a variety of congeners, some of which are readily metabolized and excreted, while others, lacking molecular sites of accessibility to oxidative enzymes, are retained in the body indefinitely, mainly in fat. The latter are generally not acutely toxic; the question of their actions during long-term storage has rarely been discussed. Our results suggested continued biological effectiveness. The matter seemed worthy of further study in light of the general contamination of human bodies throughout the industrialized world with rather high levels of PCBs, and the readiness with which these may be transmitted to offspring in milk. In the experiment reported here, a single initiating dose of DMN to newborn mice was followed by intragastric exposure to a single dose of PCBs. Neoplastic and preneoplastic lesions were measured and enumerated 4 or 7 months later, and at the same time amounts of individual PCB congeners in the bodies were quantified. The results confirmed that single doses of PCBs during the suckling period had significant, complex effects on tumor development, to some extent correlatable with retention in the bodies of two specific PCB congeners. A promoting effect of PCBs on lung tumors was demonstrated for the first time; organs other than liver must now be considered as potential targets for the action of these chemicals. This consequence of a single treatment with PCBs was concomitant with body levels of PCBs similar to those reported for the human population.

The Tumor Pathology and Pathogenesis Section (TPPS) (1) characterizes the biology and pathology of naturally occurring and experimentally induced preneoplastic and neoplastic lesions of laboratory animals; (2) uses morphologic, histochemical and ultrastructural methods to define the pathogenesis of experimental tumors; (3) develops animal models to aid in understanding causes, pathogenesis and pathology of human cancers; and (4) provides guidance, consultation and collaboration in tumor and laboratory animal pathology to investigators and scientists, in the National Cancer Institute and other U.S. Federal government agencies.

The pathology and biology of experimentally induced and naturally occurring neoplasms of rodents are characterized and compared. Pathology and histogenesis of individual tumor types are investigated with the use of serial sacrifice studies, immunocytochemistry, automated image analysis with stereology, conventional light microscopy, ultrastructure and histochemistry. Computerized image analysis of early and late induced focal proliferative lesions was used, together with stereologic techniques, to demonstrate that small focal hyperplastic lesions progressively grew in size to develop into adenomas and carcinomas. Detailed histogenesis investigations were performed for mouse, rat and hamster liver, rat thyroid gland, and mouse and rat lung.

The avidin-biotin peroxidase complex (ABC) immunocytochemical technique was further developed and evaluated for use in laboratory animals. Ninety-eight different antisera, including several monoclonal antibodies, were used to localize a variety of antigens including oncogene-associated protein products, polypeptide hormones, fetal antigens, enzymes, lysosomal proteins and cell surface glycoproteins. Several of these antisera were used for the first time on tissue sections in an effort to evaluate their specificity for oncogenesis. Problems in fixation and interpretation were solved for specific antibodies including oncogene protein products and fetal antigens

allowing us to localize these antigens in tissue sections in the various stages of carcinogenesis.

Pulmonary neoplasms in rats and mice were further characterized by histogenesis and immunocytochemical studies. Antibodies to surfactant apoprotein and Clara cell antigens were previously utilized to show that the vast majority of naturally occurring pulmonary tumors of rats and mice and tumors induced by diethylnitrosamine (DEN) and ethylnitrosourea (ENU) in mice and nitrosomethylurea (NMU) in rats were of alveolar Type II cell origin. In these model systems, no tumors of Clara cell origin have yet been identified, in contrast to the hamster.

The pathogenesis and promotion of tumors were studied using liver initiation-promotion systems in mice and rats, skin painting studies in mice, and an aged F344 rat liver model system developed in this Section. From these efforts and a review of those of other investigators, we conclude that tumor promotion can be an irreversible biological process which may require only a short period of exposure to the promoter for effective tumor promotion. For example, in the skin of SENCAR mice, we showed that after only two or four exposures to TPA, effective skin tumor promotion was seen. In addition, the tumors promoted after only short-term exposure to TPA grew progressively and did not regress after exposure to TPA was terminated. In mouse liver, the tumor promoter di(2-ethylhexyl)phthalate (DEHP) was effective as a tumor promoter after only 28 days of exposure while phenobarbital (PB) was only effective after continuous exposure.

Two widely prescribed benzodiazepine tranquilizers, diazepam and oxazepam, have been shown to promote hepatocarcinogenesis in B6C3F1 mice initiated with DEN. This finding is of special interest in view of the fact that these compounds have been found to be ineffective as liver tumor promoters in the rat (Remandet, B. et al., Fund. Appl. Toxicol. 4: 152-163, 1984). The marked and unexplained differences among rodent species in susceptibility to the promoting effects of both these classes of compounds demonstrate the impossibility of extrapolating with confidence between even closely related species, let alone from rodents to man, and emphasize the importance of studies to define the mechanisms that effect tumor promotion and presumably are related to interspecies differences in susceptibility to promoting agents.

The possible mechanisms for carcinogenesis by so-called nongenotoxic carcinogens or tumor promoters in rodent liver were investigated. Aged F344 rats, which have naturally occurring focal proliferative basophilic hepatocellular lesions, were given phenobarbital or DEHP to determine the effects on these naturally occurring foci. Phenobarbital was found to induce focal eosinophilic GGT-positive, hepatocellular foci de novo and not to promote the growth or increase the incidence of the basophilic foci. DEHP, a mitogen liver toxin and carcinogen did not induce foci or tumors or promote the natural foci. Thus, tumor promotion in rat or mouse model liver systems is not dependent on liver toxicity per se, but rather the nature of the promoter and its biological effects on target tissue cells.

The TPPS provides guidance in pathology and evaluation of carcinogenesis investigations to other scientists at the NCI, U.S. regulatory agencies, and to the National Toxicology Program. Section members have participated in government reviews of the carcinogenicity of nitrofurans, cyclamate and food, drug and cosmetic dyes and carcinogenic drugs, and as expert witnesses in government proceedings on some of these and related issues.

The Chemistry Section plans and conducts laboratory research on the chemistry of organic and inorganic carcinogens. This includes (1) investigations on mechanisms of carcinogen formation, with the aim of understanding and ultimately preventing formation of such compounds in vitro and in the environment; (2) studies on chemical reactivity of carcinogens, to identify reaction paths and products causally related to tumor formation as well as alternative pathways that may destroy carcinogens or otherwise interrupt carcinogenic reaction sequences; and (3) comparative investigations of molecular interactions between chemical carcinogens and the cells of different organs and species to identify factors potentially contributing to organ specificity and species differences in chemical carcinogenesis.

Primary attention has been given to the biological chemistry of the carcinogenic nitrosamines. Evidence has been found that the mechanism by which methylamine arises metabolically from N-nitrosodimethylamine (NDMA) is identical to that of the "denitrosation" pathway being studied in vitro. Pharmacokinetic data indicating that deuteration of the substrate may cause metabolic switching to this potentially detoxifying pathway have been obtained; if a similar means of shifting the metabolism of undeuterated NDMA away from the activating alpha-hydroxylation/methylation pathway can be found, protection of individuals from the effects of exposure to the carcinogen might be realized. Beta-hydroxylation of N-nitrosomethylethylamine (NMEA) has been shown to be a major metabolic pathway for this carcinogen, as predicted from published deuterium isotope effect studies; this process may play a key role in determining the species selectivity and organotropic effects of NMEA. Both conformers of the methanediazotate ion have been prepared as the thallium(I) derivatives, providing a new look at the chemistry of the diazohydroxides/primary nitrosamines, thought to be the ultimate alkylating intermediates in carcinogenesis, by a variety of compounds containing nitrogennitrogen bonds. Several barbiturates, hydantoins, and acylureas have been synthesized in kilogram quantities for structure-activity investigations in tumor promotion being performed in collaboration with other LCC scientists.

The Nutrition and Metabolism Section (1) investigates the effects of dietary constituents on target tissue susceptibility to chemical carcinogenesis, (2) studies mechanisms by which dietary constituents such as methyl donors and metals or their metabolites alter carcinogenic processes, and (3) investigates the role of physiologically essential divalent metals in protecting against metal carcinogenesis.

The Section has focused its interest on the role of lipotropes, methionine, choline, vitamin B_{11} and folic acid in chemical carcinogenesis. The chronic administration of diets devoid of methionine and/or choline has been shown to promote the formation of hepatocellular carcinomas in the livers of F344 rats and B6C3F1 mice initiated with DEN. Admistration of diets devoid of both methionine and choline led to the formation of metastatic hepatocellular carcinomas in both species, even in the absence of any further treatment with hepatocarcinogens. Chronic feeding of diets lacking both methionine and choline to F344 rats causes a drop in the hepatic contents of S-adenosylmethionine, an increase in its metabolic inhibitor, S-adenosylhomocysteine, and a decrease in the 5-methyldeoxycytidine contents of hepatic DNA. Chronic administration of the hepatocarcinogen, ethionine, causes similar decreases in hepatic S-adenosylmethionine and 5-methyldeoxycytidine, as well as high liver levels of the methylase inhibitor S-adenosylethionine. Further evidence that methyl insufficiency exerts a causative role in hepatocarcinogenesis in vivo is provided by the observation that tumor promotion and causation in the livers of C3H mice treated with phenobarbital are inhibited by high dietary levels of methionine.

Recent in vitro studies also provide evidence of a contributing role of methyl insufficiency in carcinogenesis. Deazaadenosine, an inhibitor of physiological methylation, transforms rat liver cells in culture, as evidenced both by growth in soft agar and by tumor formation from such cells implanted into isologous hosts. Finally, transfection of NIH 3T3 cells has been achieved with DNA isolated from hepatocellular carcinomas produced in F344 rats initiated with diethylnitrosamine and subsequently fed the methyl-deficient diets.

These results provide reasonable evidence that a physiological insufficiency of methyl donors, possibly acting via hypomethylated DNA, contributes significantly to hepatocarcinogenesis in certain rodents. Results from this and other laboratories have shown clear associations between methyl insufficiency, DNA hypomethylation and tumor formation, even in humans. Establishment of a causal relation between methyl insufficiency and tumor formation in a variety of tissues and cell types would be of major significance in understanding the etiology of cancer.

Recent results have shown that the physiological effects of carcinogenic divalent metals are often inhibited by the physiologically essential divalent metals, calcium, magnesium and zinc. Magnesium administered along with the carcinogenic metal inhibits lung adenoma formation in strain A mice by lead and nickel and sarcoma production in rats by nickel and cadmium. The inhibition by magnesium of carcinogenesis by metals appeared to result in part from the reduced uptake and retention of the carcinogenic metal in the target tissue. Calcium injection also inhibits lung adenoma induction in strain A mice by nickel and lead. However, high dietary calcium levels enhanced the carcinogenic activity of lead toward the kidneys of rats. In other studies the chemical binding of cadmium and of nickel to DNA was found to be inhibited by calcium, magnesium and zinc to an extent paralleling their inhibitory effects on cadmium and nickel carcinogenicity. These results indicate a frequent, but not universal, inhibition of metal carcinogenesis by the physiologically essential divalent metals. Such inhibition results in part from altered metabolism of the carcinogenic metal and may be associated with a decreased binding of the carcinogen to DNA. Further association between DNA and metal carcinogens was provided by studies from this Section showing a synergism between cadmium and azacytidine in inducing metallothionein in the livers of rats; in cell culture at least metallothionein induction is dependent upon gene hypomethylation. In general, the area of metals carcinogenesis can be described as lacking (1) sufficient members of research groups investigating the problem, (2) adequate model biological systems in which the effects of organic and inorganic carcinogens may be compared, and (3) reasonable evidence regarding the critical site(s) of attack of the carcinogen in the target tissue. In view of the widespread occurrence of metal carcinogens and of the great activity of some, investigations on their mode of action are important. The use of physiological metal antagonists may be expected to help specify the cellular sites of activity of the carcinogenic metals. The present studies will be expanded by examining the effects of essential metal deficiency on metal carcinogenesis.

The Ultrastructural Studies Section plans and conducts research to investigate the differentiation of potentially neoplastic epithelial cells and its relation to phenotypic expression of the neoplastic genotype with special emphasis on various cell interactions during the transformation process. Our special goals are to evaluate 1) the cellular mechanisms of neoplastic transformation of epithelial cells due to chemical carcinogens; 2) cellular mechanisms of tumor promotion in initiated epithelial cells; 3) the regulatory role of retinoids in differentiation and cell proliferation; and 4) the interaction of tumor cells with their environment. The work is organized into five projects.

The Section has continued a research program on the evaluation of cell-cell and cell-substrate changes following chemical transformation of cultured rat liver epithelial cells. In the Nutrition and Metabolism Section of the Laboratory, several sublines originating from rat liver tissue, some untransformed and some transformed by DL-ethionine, have been established and serve as models for our investigation. These sublines are of special value since low passage control cells are available as well as control cells of the same high passage as the DL-ethionine transformed cells. Our electron microscopic and immunofluorescence studies give evidence, in cultures of transformed cells, for an increase in cellsubstrate adhesion due to increases in the number of focal contacts and in the expression of fibronectin; concomitantly, a loss of cell-cell adhesion via intermediate junctions is indicated. The changed adhesion patterns in the transformed cell cultures may be defined as progressive deficiencies in cell contact interactions. Although decreased cell-substrate adhesion is commonly associated with tumorigenic transformation, our contrasting observation of decreased cell-cell contact associated with increased cell-substrate adhesion in the transformed liver epithelium makes this system a unique and valuable model for the study of the transformation process independent of a reduction in cell-substrate adhesion. We were able to show a shift in the composition of the extracellular matrix from laminin to fibronectin with transformation indicating that specific molecular interactions of adhesion are more important than adhesion per se. We also found increased fibronectin in livers of ethionine-fed rats. These results parallel the results obtained from transformed cells in culture and show the value of the cell lines as a model which related to in vivo carcinogenesis.

The rat liver cell system comprises statically transformed cells. Although the results obtained with this model are of major importance for the understanding of the transformation of these cells, they do not shed direct light on the transformation process per se. This process, in chemical carcinogenesis, is divided into at least three major successive events: initiation, promotion, and malignant conversion. During promotion, disturbance of the normal growth pattern takes place, leading eventually to overt malignancy. To elucidate promotion-dependent changes in cellular morphology we studied the effects of the tumor promoter, TPA, on initiated, promotable epithelial cells derived from mouse skin (line JB6). Our morphologic studies were complemented by investigation of the H-ras oncogene product p21 to gain new insights into functional mechanisms of cell transformation. A single TPA exposure causes only a low percentage of cells to become transformed. Moreover, TPA-induced morphologic alterations of both the cytoskeleton and the extracellular matrix are to a large extent reversible upon removal of the tumor promoter. To obtain malignant conversion with phenotypic alterations, however, both repeated cloning and repeated exposure of the cells to the promoter are necessary. Our results also provide a new finding suggesting a role of the ras gene in tumor promotion. We found the H-ras oncogene product p21 present in focus-forming cells; yet, constant presence of the protein p21 was not necessary for maintenance of the transformed state. The result represents the first observation suggesting a link between the H-ras p21 and promoter-dependent focus formation and raises the possibility that the H-ras oncogene plays a major role in certain steps during tumor promotion by singling out a selected (initiated?) group of cells and providing them with an elevated growth potential. Since we have shown that such cells can still revert to a normal growth pattern, the full expression of transformation may depend on further genetic alterations (malignant conversion).

The Section Head has been invited by the Laboratory of Chemoprevention, NCI, to join a collaborative study investigating specific developmental defects of retinoid-deficient chicken and quail embryos with the goal of understanding the molecular

mechanisms of retinoid action in modifying cell differentiation and cell proliferation. Retinoids play an important role in control of differentiation and proliferation of cells not only of epithelial origin but also of mesenchymal origin. Recently, it has been shown that retinoids are required for the formation of the vascular system system in the early avian embryo. Retinoid deficiency leads to a conspicuous absence of the extra-embryonal vascular system and failure to establish a functional circulatory system. This system of retinoid-deficient avian embryos is ideally suited to our interests in the interactions of retinoids, growth factors, and oncogenes in control of embryonic development. Oncogenes have been shown to be developmentally regulated and differentially expressed during embryogenesis in the mouse. Growth factors, as well, have been implicated in the rapid proliferation characteristic of embryonic tissue. Thus, this avian embryo system should permit study of selective (and reversible) effects of retinoids on normal development both in vitro and in vivo and permit examination of specific gene activation using techniques of genetic analysis including in situ hybridization. The successful identification of the major defect in the development of the circulatory system provides the foundation for our further examination of interactions of retinoids, growth factors, and oncogenes in control of embryonic differentiation.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP04542-13 LCC

PERIOD COVERED
October 1, 1984 to September 30, 1985

TITLE OF PROJECT (*80 characters or less. Title must fit on one line between the borders.*)
Chemistry of N-Nitroso Compounds

PRINCIPAL INVESTIGATOR (*List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	L. K. Keefer	Chief, Chemistry Section	LCC	NCI
Others:	T. Anjo	Visiting Associate	LCC	NCI
	H. S. Hu	Staff Fellow	LCC	NCI
	J. Malin	Guest Researcher	LCC	NCI

COOPERATING UNITS (*if any*) PRI, Frederick, MD (G. Lunn; E. Sansone); SK&F Labs., Philadelphia, PA (B. Mico); Hoffmann-LaRoche, Inc., Nutley, NJ (W. Garland); NJ Med. Sch., Newark, NJ (C. Yang); Universitatsspital Zurich, Zurich, Switzerland (P. Kleihues); LBI, Frederick, MD (W. Lijinsky); Clemson Univ., Clemson, SC (J. Fanning)

LAB/BRANCH
Laboratory of Comparative Carcinogenesis

SECTION
Chemistry Section

INSTITUTE AND LOCATION
NCI, NIH, Frederick, MD 21701

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
3.0	3.0	0

CHECK APPROPRIATE BOX(ES)
☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (*Use standard unreduced type. Do not exceed the space provided.*)

Primary attention has been given to the biological chemistry of the carcinogenic nitrosamines. Evidence has been found that the mechanism by which methylamine arises metabolically from N-nitrosodimethylamine (NDMA) is identical to that of the "denitrosation" pathway being studied in vitro. Pharmacokinetic data indicating that deuteration of the substrate may cause metabolic switching to this potentially detoxifying pathway have been obtained; if a similar means of shifting the metabolism of undeuterated NDMA away from the activating alpha-hydroxylation/methylation pathway can be found, protection of individuals from the effects of exposure to the carcinogen might be realized. Beta-hydroxylation of N-nitrosomethylethylamine (NMEA) has been shown to be a major metabolic pathway for this carcinogen, as predicted from published deuterium isotope effect studies; this process may play a key role in determining the species selectivity and organotropic effects of NMEA. Both conformers of the methanediazotate ion have been prepared as the thallium(I) derivatives, providing a new look at the chemistry of the diazohydroxides/primary nitrosamines thought to be the ultimate alkylating intermediates in carcinogenesis by a variety of compounds containing nitrogen-nitrogen bonds. Several barbiturates, hydantoins, and acylureas have been synthesized in kilogram quantities for structure-activity investigations in tumor promotion being performed in collaboration with other LCC scientists.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

L. K. Keefer	Chief, Chemistry Section	LCC	NCI
T. Anjo	Visiting Associate	LCC	NCI
H. Hu	Staff Fellow	LCC	NCI
J. Malin	Guest Researcher	LCC	NCI

Objectives:

Generally, to apply the methods and concepts of chemistry toward the solution of important problems in cancer research, especially by elucidating new mechanisms of formation, destruction, metabolism, and biological action of nitrosamines and related carcinogens. Specifically, (1) to establish mechanisms of nitrosamine formation so that strategies for preventing environmental contamination by these compounds can be developed; (2) to gather information on the chemistry of nitrosamine destruction so that procedures may be devised for intercepting these carcinogens before human exposure can occur; (3) to study the interactions between N-nitroso compounds and organisms exposed to them, with the aim of inferring ways of protecting victims of unavoidable nitrosamine exposure from their carcinogenic effects; (4) to characterize the fundamental physical and chemical properties of the carcinogenic N-nitroso compounds as a means of contributing to the general fund of knowledge about such materials.

Methods Employed:

The standard methods of synthetic, mechanistic, analytical, and biological chemistry have been employed in these studies.

Major Findings:

Mechanisms of NDMA metabolism

Previous work with the potent carcinogen, N-nitrosodimethylamine (NDMA), revealed a gap in our knowledge concerning its mode of biological action. In a study performed with the collaboration of Dr. Peter Swann of the Courtauld Institute in London (Swann, Mace, Angeles, and Keefer, *Carcinogenesis* 4: 821-825, 1983), we had attempted to probe the mechanistic significance of our earlier finding (Keefer, Lijinsky, and Garcia, *JNCI* 51: 299-302, 1973) that fully deuterated N-nitrosodimethylamine (i.e., $[^2H_6]$NDMA) is a less potent hepatocarcinogen than its undeuterated parent (NDMA). In Swann et al., we reported that small doses of NDMA similar in size to those ingested during the carcinogenicity study yielded 50% more methylation of liver DNA than did equimolar doses of $[^2H_6]$NDMA.

This finding correlates well with, and possibly explains, the reduced hepatocarcinogenic potency of $[^2H_6]$NDMA, but it is not consistent with certain other currently accepted principles of NDMA metabolism. There is a considerable body of evidence (as summarized, for example, in Skipper, Tomera, Wishnok, Brunengraber, and Tannenbaum, *Cancer Res.* 43: 4786-4790, 1983) that hepatic metabolism is essentially the

only significant pathway available for eliminating NDMA from the rat. This metabolism is generally considered [Preussmann and Stewart. In Searle (Ed.), Chemical Carcinogens, 2nd Edition, Vol. 2, pp. 643-828. Washington: American Chemical Society (ACS Monograph 182), 1984] to involve enzymatic C-hydroxylation to the unstable intermediate, N-nitrosomethyl(hydroxymethyl)amine (DMN-OH), which spontaneously decomposes to a powerful alkylating agent capable of methylating nucleic acids in a given organ in direct proportion to the extent of metabolism in that organ (Diaz Gomex, Swann, and Magee, Biochem. J. 164: 497-500, 1977; Pegg and Perry, Cancer Res. 41: 3128-3132, 1981). However, if it is true that metabolic hydroxylation in the liver to obligatorily methylating species is the only significant elimination pathway for NDMA, total eventual liver methylation by [$^2H_6$]NDMA should have equaled that by NDMA even if the rate of the former's metabolism were retarded several-fold, since almost all the nitrosamine must, over time, return to the liver for metabolic clearance. This difference between expected and found deuterium isotope effects on metabolic conversion to alkylating agent such that an elimination pathway not accompanied by an isotope effect and not resulting in liver alkylation was compensatorily increased in importance.

To obtain the reference rate data appropriate for a systemic inquiry into the nature of any such unknown pathways that may exist, we completed a major collaboration with Dr. Bruce A. Mico, a pharmacokineticist from Smith Kline & French Laboratories with whom we had frequently consulted on problems of this type, and Dr. William A. Garland, a Hoffmann-La Roche scientist who had developed an extremely sensitive and reliable analytical method for NDMA in blood (Garland, Holowaschenko, Kuenzig, Norkus, and Conney, Banbury Report 12: 183-196, 1982). The purpose of the collaboration was to compare the pharmacokinetics of NDMA and [$^2H_6$]NDMA at doses similar to those used to elicit liver tumors in the chronic feeding studies by following blood substrate levels. In this way, we sought to avoid reliance on indirect indicators of metabolism, as in the DNA alkylation or carbon dioxide exhalation studies. Experimentally, male Fischer 344 rats from the FCRF colony at 7.5 weeks of age were given nitrosamine bolus doses of 1.35 µmole/kg by tail vein injection and 2.02 or 4.05 µmole/kg by oral gavage. Animals were sacrificed at various time points from 2.5 to 180 min after i.v. administration or 5 to 120 min after oral dosage, and their blood was analyzed for NDMA by gas chromatography high resolution mass spectrometry. After i.v. injection, blood nitrosamine concentrations declined in an apparently biexpoential manner with terminal half-lives for NDMA and [$^2H_6$]NDMA of 10 and 12 min, respectively. The apparent total systemic blood clearances for NDMA and [$^2H_6$]NDMA were 39 and 26 ml/min/kg, respectively, and were thus, to some extent, blood flow limited. The apparent steady-state volumes of distribution were nearly identical (297 and 309 ml/kg, respectively). The areas under the curves after 2.02 and 4.05 µmole/kg oral doses were proportional to dose, indicating that metabolism was first order under the conditions of our experiment. The apparent bioavailability of NDMA was 8%, while that of [^2H-$_6$]NDMA was 21%, confirming that the normally extensive metabolism of orally administered NDMA on first pass through the liver is markedly less efficient when the substrate is deuterated. The isotope effects calculated as the ratios of first pass metabolism, total systemic clearances, bioavailabilities, and intrinsic hepatic clearances were 1.2, 1.5, 2.6 and 3.9, respectively. The isotope effect determined from blood concentrations measured after simultaneous administration of NDMA and [$^2H_6$]NDMA by steady-state infusion, each at 1.5 µmole/kg/hr, was 2.6 $\pm$ 0.9.

This study provides the first quantitative reference data on the time course of disappearance of both NDMA and fully deuterated NDMA from rat blood at low doses. It also confirms, using direct blood measurements, previous conclusions based on alkylation data (Diaz Gomez, Swann, and Magee, Biochem. J. 164: 497-500, 1977; Pegg and Perry, Cancer Res. 41: 3128-3132, 1981) that there is a substantial first pass effect on metabolism of NDMA after oral dosage, vindicating the use of alkylation data for this purpose and permitting estimation of bioavailabilities from actual blood levels. However, the observed quantitative effects of deuteration did not account for the difference between the observed and theoretical isotope effects of 1.5 and 1.0, respectively, on total 7-meG production in liver DNA; therefore, we interpret this latter result as evidence that there are at least two kinetically important mechanisms for elimination of NDMA in vivo and that deuteration of the substrate causes metabolic switching to the pathway(s) with the smaller isotope effects. Our results thus offer an alternative line of support for conclusions based on the stoichiometry of nitrogen metabolism that a significant fraction of NDMA metabolism in vivo does not proceed via the diazonium ion (Michejda, Kroeger-Koepke, Koepke, Magee, and Chu, Banbury Report 12: 69-85, 1982).

Having established another type of evidence indicating that the alpha-hydroxylation/diazonium ion production pathway is not the only metabolic route for NDMA, we considered it crucial to learn more about what the alternative mechanism(s) might be. In this effort, we undertook a collaboration with Professor C. S. Yang of the University of Medicine and Dentistry of New Jersey. Dr. Yang had devoted considerable research attention to the in vitro metabolism of nitrosamines using a variety of enzyme systems, including specially induced nitrosamine metabolizing microsomes and reconstituted enzyme systems. His effort led to important insights about the role of cytochrome P-450 enzyme systems in the metabolism of such substrates as NDMA, and further led to conclusions about which of the isozymes seem to be responsible for the activity (Yang, Tu, Koop, and Coon, Cancer Res. 45: 1140-1145, 1985, and references therein). His work also led him, however, to a mystery of his own: why should nitrite ion always be produced during the in vitro metabolism of the simple nitrosamines in a fixed proportion (about 10% in the case of NDMA) to the expected alpha-hydroxylation product, formaldehyde? Reasoning that this feature may reflect a common origin (i.e., from the same precursor) of the two products, we have begun a collaboration with him aimed at establishing the identity of this common intermediate. Specifically, we suggested that the alpha-hydroxylation product whose decomposition in aqueous medium was first shown in our Laboratory (Roller, Shimp, and Keefer, Tetrahedron Letters, 2065-2068, 1975) to produce formaldehyde, nitrogen, and methylation products might also yield, under the conditions of incubation, some (i.e., 10%) nitrite as well. If the intermediate alpha-hydroxy compound were to undergo 1,2-elimination of the elements of nitrous acid to form the species CH_3-N=CH_2, the Schiff's base corresponding to methylamine and formaldehyde, subsequent hydrolysis should produce not only formaldehyde but also methylamine.

However, the generation of nitrite from nitrosamines in vitro ("denitrosation") is formulated as proceeding with reductive cleavage of the nitrosamine to the secondary amine (Preussmann and Stewart, loc. cit., pages 684 and 686; Appel, Schrenk, Schwarz, Mahr, and Kunz, Cancer Lett. 9: 13-20, 1980). The first goal of our collaboration with Prof. Yang, therefore, was to measure the relative proportion of primary amine (if any) among the organic products of NDMA denitrosation.

Incubations of NDMA with acetone- or ethanol-induced rat liver microsomes were performed by Prof. Yang and colleagues in Newark. They determined both formaldehyde and nitrite colorimetrically in each sample and sent the remainder to us for the amine analysis, which was performed using a modification of a published method (Koga, Akiyama, and Shinohara, Bunseki Kagaku 30: 745-750, 1981). The preliminary results yield several important conclusions. Dimethylamine appears not to be produced in significant quantity during incubation, but is carried through as a contaminant of the starting nitrosamine. Methylamine, on the other hand, is definitely produced by metabolic reaction; we are especially confident of this conclusion because the derivatized amines gave rise to clearly resolved peaks in the gas chromatogram and the identity and homogeneity of the peak due to the methylamine derivative were confirmed by mass spectrometry. Quantitatively, the yields of methylamine and nitrite appear to be nearly equimolar in these incubations.

While these experiments supported the hypothesis that the alpha-hydroxy compound was the common precursor for both nitrite and formaldehyde, another experiment we performed to confirm this did not. Instead of incubating NDMA with induced microsomes, we hydrolyzed the E alpha-acetoxy compound (E-NDMA-OAc) with hog liver esterase as well as microsomes. The amounts of formaldehyde, nitrite, and methylamine present at the end of incubation were 292.1, 1.1, and 1.2 nmole/ml, respectively. Even if the methylamine were entirely produced on incubation rather than present at the outset as a contaminant (a possibility that has not as yet been checked), its quantity was only 5% of that produced during the microsomal metabolism of NDMA. Thus, the E-NDMA-OH produced on hydrolysis of E-NDMA-OAc appears not to be the common precursor of nitrite and formaldehyde, in contrast to our original hypothesis. But if E-NDMA-OH is not the metabolic intermediate we are seeking, what is? A possible mechanism that could explain the results is heteroatom oxidation.

If NDMA were converted to a "nitrosamine N-oxide" by the metabolizing enzymes, such a species could conceivably rearrange to a $Me_2N-O-N=O$ intermediate also capable of eliminating HONO to form the methylamine-formaldehyde Schiff's base. Our interest in testing this attractive possibility was stymied initially by our inability to find any data on nitrosamine N-oxides in the literature; presumably they are highly unstable and cannot be directly observed. Recognizing that the same structures might be achievable by indirect reactions, we have been studying the nitrosation of N,N-dimethylhydroxylamine. The initial product of N-nitrosation of the hydroxylamine should be the nitrosamine N-oxide, while that of O-nitrosation (of N-oxide rearrangement) should be the $Me_2N-O-N=O$ species. So far we have identified formaldehyde as a product of N,N-dimethylhydroxylamine nitrosation, but methylamine was absent when the reaction of the hydroxylamine hydrochloride at 1 M with equimolar sodium nitrite in deuterium oxide finished bubbling and was analyzed by ^{13}C-NMR. It is not yet clear whether methylamine is simply not formed in this reaction or whether it is formed and quickly deaminated to methanol in the presence of acidified nitrite. Interestingly, methylamine was unstable in this medium and methanol was identified as a constituent of the final reaction mixture. Thus, it is possible that the N-oxidation mechanism postulated is the alternate NDMA metabolism pathway we are seeking to characterize, but many questions remain to be answered before an informed decision on this point can be made. For example, a third product identified in the hydroxylamine nitrosation mixture was formate, raising the possibility that the methanol might have been formed by Cannizzarro disproportionation of the formaldehyde rather than by methylamine deamination. Another problem is that there is a fourth major product in the nitrosation mixture that remains unidentified.

Studies currently in progress are aimed at elucidating the products and mechanism of N,N-dimethylhydroxylamine nitrosation.

A second possible explanation as to why E-NDMA-OAc gave rise to a solution containing only 5% of the expected quantities of methylamine and nitrite on esterase hydrolysis is that the alpha-hydroxy nitrosamine produced during enzymatic oxidation of NDMA may be different from the one produced on hydrolysis of the ester. Unsymmetrical nitrosamines such as NDMA-OH generally have enough double bond character in their nitrogen-nitrogen linkages that Z- and E-isomers are distinguishable. Enzymatic hydroxylation of NDMA at the carbon atom anti to the nitroso oxygen would give E-NDMA-OH, the isomer that is presumably produced on NDMA-OAc hydrolysis and decomposes to formaldehyde, nitrogen gas, and a methylating agent. But hydroxylation at the carbon atom syn to the nitroso oxygen would produce Z-NDMA-OH, a species that has not yet been isolated and whose chemistry has yet to be explored. To the extent that Z-NDMA-OH might decompose by 1,2-elimination of HONO to form the methylamine-formaldehyde Schiff's base, the products of microsomal attack on NDMA could be explained. It is perhaps coincidental that the amount of nitrite and methylamine found after esterase hydrolysis of the alpha-acetoxy compound--5% of the formaldehyde yield--is equal to the fraction of the alpha-acetoxy compound present as the Z conformer in the equilibrium mixture.

We have begun to explore the decomposition reactions of Z-alpha-hydroxy-nitrosamines to learn whether the methylamine and nitrite could be produced by decomposition of this intermediate. One strategy for accomplishing this is to separate the Z and E conformers of an alpha-hydroperoxy nitrosamine, deoxygenate them individually to the respective pure alpha-hydroxy compounds (Mochizuki, Anjo, and Okada, Tetrahedron Lett. 21: 3693-3696, 1980), and study their solvolytic behavior individually. So far, we have succeeded in achieving partial resolution of the Z and E conformers corresponding to sec-butyl(alpha-hydroperoxymethyl)nitrosamine by high pressure liquid chromatography (HPLC). The two conformers are inseparable at room temperature, but some separation was possible by performing the chromatography at temperatures near 0°C. Complete separation may prove possible in planned HPLC work at even lower temperatures. Similar procedures led to complete resolution of the corresponding Z and E acetoxy compounds, and it is possible that these could be hydrolyzed sufficiently rapidly by treatment with massive amounts of esterase that the Z and E alpha-hydroxy compounds could be characterized satisfactorily. Future work will focus on the methyl, rather than sec-butyl, derivatives so that conclusions about the chemistry of the alpha-hydroxy compound produced in the above incubations can be made directly, rather than by inference from structurally related nitroso compounds. One problem in dealing with the methyl compounds is that the proportion of Z isomer in the equilibrium mixture is much smaller than that for the sec-butyl derivative. This could make it more difficult to collect adequate amounts for the solvolysis studies, but it is possible that the proportion of Z isomer can be enriched by photoisomerization methods discovered earlier (Michejda, Davidson, and Keefer, J. Chem. Soc., Chem. Commun.: 633-634, 1976).

Another approach we have devised for studying the possibility that the Z alpha-hydroxy derivative of NDMA is the one produced by enzymatic oxidation involves the stereoselective synthesis of a specifically labeled isotopic variant of NDMA in which the two magnetically and stereochemically nonequivalent methyl groups are made permanently distinguishable by isotopic tracers. To accomplish this, we have developed the stereospecific preparation of the two deuterium labeled compounds, Z- and

E- N-nitrosomethyl([C^2H_3]methyl)amine ([$^2H_3$]NDMA). This was made possible by the discovery, in our Laboratory, of a new nitrosamine synthon, thallium(I) antimethanediazotate. We have now completed experimental work for a paper in preparation describing the physicochemical properties of this interesting material, as well as its use for the preparation of novel N-nitroso compounds. Unlike the alkali metal alkanediazotates, the thallium derivative is highly crystalline, melts reversibly at a characteristic temperature, is easily obtained in highly pure form, and is very soluble in chloroform and related nonpolar solvents. Another interesting property of this compound is that it shows unusual symptoms of covalency in the bonding between the thallium atom and the diazotate moiety.

As one demonstration of the preparative utility of this novel synthon, which can be considered to be the thallium(I) derivative of the primary nitrosamines (diazohydroxides) written as key alkylating intermediates in most formulations of activation pathways in carcinogenesis by N-nitroso compounds, one deuterated compound desired for the stereochemical studies (Z-[$^2H_3$]NDMA) was synthesized merely by dissolving the diazotate in cold [$^2H_3$]methyl iodide; after removing the solid products and excess solvent following approximately one hour of reaction at 0°, the product was purified using low temperature HPLC. N-Nitrosomethylbenzylamine was similarly prepared from benzyl bromide predominantly in its energetically less favored conformation (i.e., as the Z isomer). The potential significance of the E-thallium derivative was further demonstrated by preparing some quaternary ammonium and other onium salts of the anti-methanediazotate anion. We have also succeeded in characterizing, indirectly, the thallium(I) Z-methanediazotate, but it has so far defied isolation, and could be identified only by trapping it from the low temperature synthesis reaction mixture by addition of [$^2H_3$]methyl iodide, whereupon E-[$^2H_3$]NDMA was produced in good yield.

Before leaving the subject of NDMA metabolism, another finding should be mentioned. In our first pharmacokinetic experiments with this compound, we anesthetized the animals with diethyl ether before dosage. The results suggested that the ether was interfering with NDMA metabolism. Noting a previous report that urinary excretion of NDMA was increased when the carcinogen was administered under ether anesthesia (Spiegelhalder, Eisenbrand, and Preussmann, IARC Scientific Publications 41: 443-449, 1983) and recognizing that some important contributions to the literature of nitrosamines mention having used ether treatment in their experimental descriptions, we decided to quantify the effect pharmacokinetically. Twenty-six 6-week-old male Fischer rats were exposed to ether vapor until their righting reflex was lost (approximately 2 min). The animals were removed from the ether, and NDMA was immediately administered by i.v. bolus injection at a dose of 300 μg/kg via a cannula surgically inserted into the jugular vein 20 hr earlier. A second group of 28 rats received injections of NDMA in an identical manner but without ether exposure. Blood collected from these animals was analyzed for NDMA by gas chromatography-mass spectrometry. In the unanesthetized animals, blood levels of NDMA declined with a half-life of 11 min; by contrast, essentially constant blood levels of NDMA were observed in ether-treated animals for 120 min after removal from the anesthetic. The apparent total systemic clearance for the 5 hr experiment was reduced from 43 ml/min/kg without ether to 5 ml/min/kg with ether. The results demonstrate that exposure to ether is capable of suppressing almost totally the in vivo metabolism of NDMA and that the inhibitory effect persists long after the animals have been removed from the anesthetic. We conclude that those contemplating the collection or interpretation of data on nitrosamine metabolism be particularly mindful of the

potential for complications that may be encountered when exposure to diethyl ether is included in the protocol. The inhibition was much more pronounced in our NDMA metabolism study than was observed in previous investigations with other drugs.

Origins of species differences and organotropism in NMEA carcinogenesis

As work aimed at improving our understanding of NDMA's metabolic reactivity progresses, extensions to closely related species are being undertaken. Among the most interesting of these is the relatively little investigated 3-carbon homolog, N-nitrosomethylethylamine (NMEA), whose biological properties suggest that it may hold some important clues about the nature of organ and species specificity in carcinogenesis. For example, deuterium isotope effect studies have revealed that deuteration of the beta-methyl group shifts its organotropism from the liver toward increasing activity in esophagus (Lijinsky and Reuber, Cancer Res. 40: 19-21, 1980). This result strongly indicates that a metabolic pathway involving the beta-carbon atom may be a primary determinant of the compound's carcinogenic action.

We have begun two major collaborations aimed at determining the full mechanistic significance of this finding. In the first, we are working with Drs. L. M. Anderson (LCC) and H. Issaq (Program Resources, Inc.) and colleagues to characterize the pharmacokinetics of NMEA. One hypothesis to be tested is that beta-deuteration slows some first-pass metabolic reaction enough that relatively more of the unchanged nitrosamine escapes into the general circulation to produce tumors in susceptible distal organs. Because hydroxylation is a key initial step in the metabolism of many nitrosamines [Preussmann and Stewart. In Searle (Ed.), Chemical Carcinogens, 2nd edition, Vol. 2, pp. 643-828. Washington: American Chemical Society (ACS Monograph 182), 1984], we postulated more specifically that the results of Lijinsky and Reuber implied the existence of beta-hydroxylation as a major pathway for first-pass metabolism of NMEA.

There is currently no suggestion in the literature that metabolic beta-hydroxylation of NMEA occurs at all, so a pilot study was undertaken to determine whether any of the beta-hydroxylation product, N-nitrosomethyl(2-hydroxyethyl)amine (NMHA), could be detected. A single male Fischer rat was injected intravenously with [^{14}C]NMEA via the tail vein without anesthesia. A blood sample was collected 5 min later by retro-orbital puncture and immediately mixed with a solution containing known quantities of both NMEA and NMHA. The solution was then analyzed by HPLC. The blood sample was found to contain at least two major radioactive components: one corresponding in retention time to NMEA, the other to NMHA.

This demonstration that beta-hydroxylation is indeed a major metabolic pathway for NMEA in vivo, as predicted, is being followed up by comprehensive pharmacokinetic studies now underway. Preliminary data show that the i.v. clearance for low doses of NMEA is largely blood flow limited, leading to the prediction that beta-hydroxylation should be even more extensive after oral administration. These pharmacokinetic studies have been greatly facilitated by the use of an analytical approach patterned very closely after that of Gough, Webb and Swann (Fd. Chem. Toxic. 21: 151-156, 1983). This isotope dilution-HPLC method has proven especially sensitive, permitting repeated sampling from the same animal after low doses of the carcinogen. It is hoped that full details of these studies will be available soon.

In the second collaboration, the comparative metabolism of NMEA in the various organs of rat is being examined by following alkylation of DNA. In collaboration with Dr. V. Nelson of Program Resources, Inc., we have supplied Drs. P. Kleihues of the Universitätsspital Zürich and W. Lijinsky of Litton Bionetics, Inc., with ethyl-labeled [^{14}C]NMEA of high specific activity, and they have studied the relative extents of ethylation and methylation as well as of carbon dioxide generation in Fischer rats. Adult males received single i.p. or oral doses (4.4 mg/ kg) of NMEA labeled in the methyl or ethyl position. The animals were killed 4 hours later and the DNA of various organs was analyzed by Sephasorb chromatography following hydrolysis in 0.1 M hydrochloric acid. Concentrations of 7-meG in hepatic DNA were 170-200 times higher than those of 7-ethylguanine, suggesting that alpha-hydroxylation of NMEA occurs primarily at the ethyl group in vivo. Concentrations of 7-meG in liver were approximately 15 times higher than in kidney, 100 times higher than in esophagus, and 200 times higher than in lung. Very significantly, radioactivity traces of DNA hydrolysates from animals treated with ethyl-labeled NMEA contained 4 peaks, while those receiving methyl-labeled material showed only the expected peaks due to O^6- and 7-meG. The remaining 2 peaks have been shown to coelute with authentic 7-(2-hydroxyethyl)guanine and O^6-(2-hydroxyethyl)guanine. Thus, evidence for significant beta-hydroxylation of NMEA was obtained from the alkylation studies as well as from the pharmacokinetic investigations described above.

The next step will be to subject [^{14}C]NMEA having the beta-methyl group completely deuterated to similar experiments, the hypothesis being that beta-deuteration should decrease the amount of observable beta-hydroxylation.

Extension of these initial pharmacokinetic and alkylation studies in a different direction may also provide important insights into the origins of species differences in NMEA tumorigenesis. As a liver carcinogen in hamsters, NMEA is intermediate in potency between NDMA and N-nitrosodiethylamine (NDEA), as might be expected for a compound that can be considered a structural hybrid of the latter two nitrosamines; in Fischer rats, however, NMEA is a much weaker carcinogen than either of its symmetrical counterparts (W. Lijinsky, unpublished results). By repeating the abovementioned metabolism studies in hamsters, we can test the hypothesis suggested by the work of Lijinsky that rat liver is less able to activate (and/or better able to deactivate) NMEA relative to NDMA and NDEA than hamster liver, and that these differences between the rats' and hamsters' relative ability to metabolize the three carcinogens account for the species variations observed.

Mechanisms of nitrosamine formation

Twenty-five years ago, it was believed that nitrosamines could form only in the presence of acid (Ridd, Quart. Rev. 15: 418-441, 1961). During the last decade, powerful new analytical methodology revealed the presence of nitrosamines in several media with no history of exposure to acid. As one example, some commercial cutting fluid concentrates that were alkaline mixtures of nitrite with amines were shown to contain a carcinogenic N-nitroso compound in concentrations as high as 1-2% (Fan, Morrison, Rounbehler, Ross, Fine, Miles, and Sen, Science 196: 70-71, 1977). Reasoning that this finding implied the existence of some previously uncharacterized mechanism(s) of nitrosamine formation, we set about to learn how nitrosamines might arise in nonacidic media. We soon found that certain carbonyl compounds could replace the hydrogen ion as electrophilic accelerators of the N-nitrosation reaction (Keefer and Roller, Science, 181: 1245-1247, 1973). Reasoning further that if electrophiles

such as the hydrogen ion and formaldehyde could facilitate nitrosamine-forming reactions, then electrophilic transition metal centers such as those of the active sites of some of nature's more important enzyme catalysts might be especially effective accelerators of nitrite-amine reactions. We then tested certain metal complexes for their activity in this regard. We found preliminary evidence for several distinct roles played by electrophilic metal complexes in N-nitrosation reactions (Croisy, Fanning, Keefer, Slavin, and Uhm, IARC Scientific Publications 31: 83-93, 1980) and established further, using some simulated cutting fluids, that such complexes may well be important in the environmentally significant formation of nitrosamines (Loeppky, Hansen, and Keefer, Fd. Chem. Toxic. 21: 607-613, 1983). We are now working on definitive characterization of several of these mechanisms with the aim of publishing comprehensive accounts thereof for the chemical literature. In the case of one of these reaction types, the catalytically active hexacyanoferrate complexes (ferricyanide and ferrocyanide) had been shown to induce nitrosamine formation in nitrite-amine mixtures at pH 9 at roughly the same rates as in the formaldehyde catalyzed reaction described earlier (Keefer and Roller, loc. cit.).

We have now discovered that a commercially available salt containing the pentacyanoiron(II)ammine ion is orders of magnitude more active catalytically than any of the electrophilic accelerators of N-nitrosation reactions we had characterized previously. In a typical reaction, solutions of 1 M nitrite, 1 M morpholine, and 0.25 M in iron complex produced N-nitrosomorpholine (NMOR) in 28% yield 2.5 hr after mixing at 25%. By contrast 0.25 M ferricyanide ion gave 0.2% NMOR under these conditions, while no detectable NMOR (<0.01%) was found when ferrocyanide was used.

It may be that this system has value as a biomimetic model of certain enzymatic processes. Tannenbaum and colleagues earlier found that human saliva, known to contain both nitrite and thiocyanate, is capable of converting morpholine added to it to both N-nitrosomorpholine (Tannenbaum, Archer, Wishnok, and Bishop, JNCI 60: 251-253, 1978) and N-cyanomorpholine (Wishnok and Tannenbaum, Science 191: 1179-1180, 1976). In duplicating the ability of human saliva to induce reaction between morpholine and nitrite (as well as morpholine and thiocyanate), the cyanoiron complexes may provide a glimpse into the chemical features surrounding the active site of the salivary enzyme(s) presumably responsible for the nitrosamine and cyanamide formation characterized in the MIT work. Yields in some of these reactions are so high that they may be of potential synthetic utility.

Chemical synthesis research

We have discovered, in collaboration with Drs. George Lunn and E. B. Sansone of Program Resources, Inc., that commercially available titanium trichloride solution rapidly and conveniently converts nitrosamines to the corresponding unsymmetrical hydrazines. The synthetic utility of this procedure has just been published (Lunn, Sansone, and Keefer, J. Org. Chem. 49: 3470-3473, 1984). We have also demonstrated that addition of aluminum-nickel alloy to alkaline solutions of a wide variety of compounds containing nitrogen-nitrogen or nitrogen-oxygen bonds quantitatively and reliably converts them to the corresponding amines. The method has been successfully applied to more than 12 different functional groups; since many potent carcinogen types include N-N or N-O bonds in their structures, the simplicity and generality of this quantitative reduction procedure can be recommended as a general approach to the problem of degrading chemical wastes from carcinogen research projects. The method may also be of value as a synthetic tool, e.g., for use in elucidating the

structures of natural products containing such bonds (hydrazides, azoxy compounds, etc.) as well as in demasking of reversed polarity amine synthons. Other synthetic chemistry projects include preparation of: four barbiturates that were not commercially available but were needed for tumor promotion structure-activity investigations underway in LCC; a sample of mono(2-ethylhexyl)phthalate for the same purpose; and formaldehyde and formate ion labeled with both deuterium and carbon-14 for a collaboration with Professor T. Baillie of the University of Washington aimed at studying the deuterium isotope effect on the conversion of the initial NDMA metabolism products to carbon dioxide.

Significance to Biomedical Research and the Program of the Institute:

The primary goal of this project is to contribute toward the understanding of carcinogenesis mechanisms in enough detail that means of interrupting this sequence of molecular events might systematically be inferred. Emphasis is on the nitrosamines, which appear to be largely anthropogenic in origin. As a consequence, the most economical approach to preventing carcinogenesis by these compounds from the public health point of view is to eliminate exposure to them. For this reason, the mechanisms of nitrosamine formation are being studied with the hope that procedures for suppressing their formation in the environment and in vivo can be established. For similar reasons, we are also studying the reactivity of nitrosamines and related carcinogens on the grounds that human exposure can also be prevented if carcinogens, once formed, can be destroyed before exposure can occur. Finally, studies of the biological chemistry of carcinogenesis in animal models are being conducted with the aim of inferring means of protecting individuals from the carcinogenic consequences of unavoidable exposure. The basic research leads that arise from the above three targeted research goals are also being pursued as a means of contributing to general knowledge about such compounds. Specifically, the new NDMA metabolism pathway we are attempting to characterize could potentially lead to a very useful strategy for cancer prevention. If the mechanism does represent a detoxifying route that proceeds in competition with the activation pathway already characterized, additional studies could reveal a means for increasing the former's importance at the expense of the latter, perhaps with marked suppression of NDMA's carcinogenic effects. In examining the origins of species (and organ) specificity in the action of NMEA and related carcinogens, we may be able to shed light on the mechanisms' less vulnerable species (or organs) used to protect themselves from tumor induction after carcinogen exposures that are harmful to susceptible species (or organs). Finally, our discovery of a transition metal complex that is a highly effective catalyst of nitrosamine formation in nonacidic nitriteamine mixtures suggests a possible role for related complexes in environmental or in vivo nitrosation. In fact, this type of mechanism could prove to be a more important determinant of human exposure to nitrosamines than classical, acid-catalyzed nitrosation, since the latter process is second order in nitrite and thus of rapidly diminishing significance at decreasing nitrite concentrations.

Proposed Course:

One of our first priorities in the coming months will be to characterize as fully as possible the mechanism by which NDMA breaks down metabolically to produce methylamine and nitrite, since this pathway may represent a detoxification mechanism that could conceivably be used to advantage in protecting organisms from NDMA exposure. The relative importance of C- vs N-oxidation will be assessed by measuring the deuterium

isotope effect on the formaldehyde/methylamine ratio in vitro. If C-oxidation is implicated in methylamine formation, every effort will be made to characterize the decomposition pathways for the Z-hydroxy compounds to determine whether this might be the common intermediate being sought. It will also be extremely important to determine the quantitative significance of the new pathway in the in vivo metabolism of NDMA. Methylamine is known to be excreted in the urine of rats that have received NDMA. We plan to quantify the production of this material through a complete pharmacokinetic analysis.

Our major long-term research emphasis will be on determining the origins of species differences and organotropism in NMEA carcinogenesis. After confirming the production of NMHA as a primary metabolite of NMEA in vivo, detailed studies of the time course of disappearance of NMEA from blood will be conducted. Rates of appearance and disappearance of its major metabolites will also be determined. Clearances will be calculated from these data, as will biological half-lives and other pharmacokinetic parameters. Comparison of data from i.v. vs oral administration will permit calculation of bioavailabilities. The hypothesis that the differing relative susceptibilities of rats and hamsters to NMEA carcinogenesis is due to pharmacokinetic idiosyncrasies of the two systems will then be tested. We anticipate that the Fischer rat will beta-oxidize NMEA on first pass through the liver after oral dose to a much larger extent that it metabolizes NDEA, but that the bioavailabilities of NMEA and NDEA will prove much more similar in the hamster. Regarding the shift toward esophageal carcinogenicity on beta-deuteration of NMEA, we intend to examine the origins of the organotropic effect by directly comparing NMEA with beta-deuterated NMEA pharmacokinetically in the rat after i.v. and oral administration. We predict that the deuterated compound will have a much greater bioavailability, which would account for its greater systemic activity.

A substantial effort will also be devoted to a full characterization of the several distinct mechanisms for electrophilic acceleration of nitrosamine formation we have discovered including that governing the powerfully catalytic pentacyanoiron(II)amine ion as an inducer of nitrosamine formation in nitrite-amine mixtures. Additional barbiturates, hydantoins, and related substances will be synthesized in kilogram quantities for tests of structure-activity relationships among tumor promoters in collaboration with other LCC staff members.

Publications:

Lunn, G., Sansone, E.B., Andrews, A.W., Castegnaro, M., Malaveille, C., Michelson, J., Brouet, I. and Keefer, L.K.: Destruction of carcinogenic and mutagenic N-nitrosamides in laboratory wastes. In O'Neill, I.K., von Borstel, R.C., Miller, C.T., Long, J. and Bartsch, H. (Eds.). IARC Sci. Pub. No. 57, 1984, pp. 387-398.

Lunn, G., Sansone, E.B. and Keefer, L.K.: Reduction of nitrosamines with aqueous titanium trichloride: convenient preparation of aliphatic hydrazines. J. Org. Chem. 49: 3470-3473, 1984.

Kroeger-Koepke, M.B., Michejda, C.J., Roller, P.P. and Keefer, L.K.: On trapping alkylating intermediates with 3,4-dichlorobenzenethiol during in vitro metabolism of nitrosamines. Cancer Res. 1985. (In Press)

Ward, J.M., Diwan, B.A., Ohshima, M., Hu, H., Schuller, H.M. and Rice, J.M.: Tumor initiating and promoting activities of di(2-ethylhexyl)phthalate in vivo and in vitro. Environ. Hlth. Perspect. (In Press)

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP04580-11 LCC

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT (*80 characters or less. Title must fit on one line between the borders.*)

The Role of Lipotropes in Carcinogenesis

PRINCIPAL INVESTIGATOR (*List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	L. A. Poirier	Chief, Nutrition and Metabolism Section	LCC	NCI
Others:	M. J. Wilson	Chemist	LCC	NCI
	P. T. Allen	Microbiologist	LCC	NCI
	J. M. Ward	Chief, Tumor Pathology and Pathogenesis Section	LCC	NCI

COOPERATING UNITS (*if any*)

Hotel-Dieu de Quebec, Quebec, Canada (Dr. Luc Belanger)
McArdle Laboratory, Madison, WI (Dr. H. Pitot)

LAB/BRANCH

Laboratory of Comparative Carcinogenesis

SECTION

Nutrition and Metabolism Section

INSTITUTE AND LOCATION

NCI, NIH, Frederick, Maryland 21701

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.5	2.0	0.5

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (*Use standard unreduced type. Do not exceed the space provided.*)

The mechanisms responsible for the alteration of chemical carcinogenesis by the dietary lipotropes, choline, methionine, folic acid and vitamin B-12, have been studied. The metabolism and carcinogenic activity of ethionine in different species is being compared. Correlations between the tissue levels of the physiological methyl donor S-adenosylmethionine, its chief metabolic inhibitor, S-adenosylhomocysteine, and 5-methylcytosine in animals treated with carcinogens, liver tumor promoters and methyl-deficient diets are being determined. Using standard bioassays the effects of (1) the length of time of dietary methyl deprivation, (2) the interaction between methyl deprivation and liver tumor promoters, and (3) deficiencies of other essential nutrients on hepatocarcinogenesis are under investigation. The effects of carcinogens and methylase inhibitors on the general and specific methylation of DNA in target tissues are examined.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

L. A. Poirier	Chief, Nutrition and Metabolism Section	LCC	NCI
M. J. Wilson	Chemist	LCC	NCI
P. T. Allen	Microbiologist	LCC	NCI
J. M. Ward	Chief, Tumor Pathology and Pathogenesis Section	LCC	NCI

Objectives:

To determine the mechanism(s) by which physiological methyl deprivation produces liver carcinomas. To determine the extent to which methyl deprivation contributes to carcinogenesis in extrahepatic tissues.

Methods Employed:

The effects of carcinogenesis in rodents of dietary regimes altering the in vivo bioavailability of the chief physiological methyl donor, S-adenosylmethionine, are investigated. The dietary components varied to include the methyl group-providing compounds, methionine and choline; the vitamins responsible for methyl group biosynthesis, folic acid and vitamin B_{12}; and the methionine antagonist, ethionine.

Both complete and two-stage carcinogenesis studies are employed. The early histological events associated with hepatocarcinogenesis are monitored by light microscopy using specialized stains. Tissue levels of S-adenosylmethionine, S-adenosylethionine, S-adenosylhomocysteine, 5-methyldeoxycytidine in DNA, and polyamines are determined using appropriate combinations of HPLC and chromatographic systems developed in this Laboratory, as well as of standard spectrophotometric and radioisotopic techniques.

The cellular activities of the enzymes, DNA methylase and ornithine decarboxylase are determined by published methods. Serum levels of alphafetoprotein in rats undergoing carcinogenesis by methyl deprivation, and the transfecting activity of DNA from tumors arising in methyl-deficient rats are determined by collaborative studies with other groups.

Major Findings:

The pursuit of this project has led to the major observation that dietary methyl deprivation alone causes liver cancer in male F344 rats and B6C3F1 mice. The chronic administration of methionine- and choline-deficient diets produced a high incidence of liver cancer in both initiated and uninitiated animals. The administration of a severely methyl-deficient diet for as short a period as 15 weeks is sufficient to induce tumors in rats sacrificed at one year. Methyl deprivation, even in uninitiated rats, leads to the formation of preneoplastic lesions, such as enzyme-altered foci and elevated levels of serum alphafetoprotein commonly seen during hepatocarcinogenesis by chemicals. In a recent study a slight but significant elevation in the incidence of squamous papillomas has been seen in the forestomachs of rats fed a diet deficient in vitamin B_{12} as well as in methionine and choline. Quantitative studies show that at very low dietary levels of methionine and choline, the latter

is more protective against liver tumor promotion by methyl deficiency than is the former.

In general the biological effects of methyl deprivation and ethionine administration can be correlated with their biochemical effects in different tissues. For example, of all rat organs studied, the liver suffered the greatest decline in the ratio of S-adenosylmethionine to S-adenosylethionine (in ethionine-fed rats) or to the physiological methylase inhibitor, S-adenosylhomocysteine (in choline- and methionine-deficient animals). In both cases such decreases were accompanied by a significant decline in the 5-methyldeoxycytidine content in hepatic DNA. The chronic administration of a methionine- and choline-devoid diet to C3H mice, which are resistant to the hepatocarcinogenic effects of methyl deprivation, produced only slight alterations in the ratios of hepatic S-adenosylmethionine to S-adenosylhomocysteine and no significant change in the proportion of 5-methyldeoxycytidine in hepatic DNA. These results provide good evidence that dietary methyl insufficiency results in hypomethylated DNA and, at least under some conditions, plays a major role in hepatocarcinogenesis.

Significance to Biomedical Research and the Program of the Institute:

One of the basic aims of the National Cancer Institute is the prevention of cancer by a delineation of the mechanism by which cancers are induced. The reasons for studying the role of physiological methyl donors in carcinogenesis are both practical and theoretical. In practice, several physiological conditions associated with an elevated risk of cancer formation in humans are also accompanied by an abnormal stress on the body's pool of methyl donors. These include 1) High fat intake. High fat diets increase the metabolic requirements for methionine and choline (hence the term lipotropes). 2) Familial polyposis. Biologically normal fibroblasts from colon cancer patients with this disease have an increased demand for methionine compared to the fibroblasts from their disease-free relatives. 3) Tyrosinemia. Patients born with this genetic disease have high serum levels of alphafetoprotein, develop a high incidence of liver carcinoma, and have a defective biosynthesis of S-adenosylmethionine. 4) Liver cancer in certain African populations. This disease has been associated with an elevated aflatoxin intake, hepatitis and a low protein, and thus low methionine, intake. The theoretical reasons for studying methyl deprivation in carcinogenesis are centered on indirect evidence implicating hypomethylation, particularly of DNA, in cancer causation. This includes the observations that a) the chronic administration of several hepatocarcinogens and liver tumor promoters decreases the hepatic levels of S-adenosylmethionine in rats; b) specific genes from several human and experimental tumors are hypomethylated compared to the same genes in the corresponding normal tissues; c) azacytidine, an inhibitor of DNA methylation, is tumorigenic in several rodent tissues; d) S-adenosylethionine, a major metabolite of the hepatocarcinogen, ethionine, and an effective inhibitor of DNA methylation, is a cell transformant. The present studies provide good evidence that physiological methyl insufficiency under specific conditions plays a major role in hepatocarcinogenesis in rodents. Successful generalization of the hypothesis that methyl insufficiency or hypomethylation is a major contributing factor to carcinogenesis would provide i) screening methods for populations at risk for specific types of cancer, ii) the prospect of early intervention to minimize such risks, and iii) an alternate mechanism to the common model systems requiring exogenous agents to produce cancer.

Proposed Course:

This project is an integrated approach to an understanding of the mechanism by which and the extent to which physiological methyl deprivation causes cancer. Future studies will test the hypothesis that systems or agents which favor hypomethylation in vivo will enhance carcinogenesis. The effects of physiological methyl donors on carcinogenesis will be extended to include other dietary modifications known to alter the availability of S-adenosylmethionine in vivo, other carcinogens and antagonists of methionine, and other tissues and species. In particular, more direct evidence for the involvement of S-adenosylmethionine and of 5-methylcytosine in carcinogenesis will be sought. Dose-response studies on the formation of enzyme-altered foci as a function of methyl content in the diet will be performed. Studies will be undertaken to determine the extent of methylation of specific genes and the transfecting activity of DNA from the liver tumors obtained in methyl-deficient animals. Alternate hypotheses to DNA hypomethylation as the mechanism of carcinogenesis by methyl deprivation will be tested by the long-term feeding of diets deficient in other essential nutrients, or supplemented with antioxidants, and by searching for abnormal bases in the hepatic DNA of methyl-deficient animals.

Publications:

Hoover, K.L., Lynch, P.H. and Poirier, L.A.: Profound influence of short-term severe methionine, choline, vitamin B_{12}, and folate deficiency and hepatocarcinogenesis in rats injected with a single low dose of diethylnitrosamine. JNCI 73: 1327-1336, 1984.

Shivapurkar, N. and Poirier, L.A.: Levels of S-adenosylmethionine and S-adenosylethionine in four different tissues of male weanling rats during subchronic feeding of DL-ethionine. Biochem. Pharmacol. 34: 373-375, 1984.

Shivapurkar, N., Wilson, M.J. and Poirier, L.A.: Hypomethylation of DNA in ethionine-fed rats. Carcinogenesis 5: 989-992, 1984.

Wilson, M.J., Shivapurkar, N. and Poirier, L.A.: Hypomethylation of hepatic nuclear DNA in rats fed with a carcinogenic methyl-deficient diet. Biochem. J. 218: 987-990, 1984.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP04582-10 LCC

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Metal Interactions in Carcinogenesis

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	L. A. Poirier	Chief, Nutrition and Metabolism Section	LCC	NCI
Others:	K. S. Kasprzak	Visiting Scientist	LCC	NCI
	M. P. Waalkes	Staff Fellow	LCC	NCI
	M. J. Wilson	Chemist	LCC	NCI

COOPERATING UNITS (if any)

Program Resources, Inc., Frederick, MD (C. Riggs)
Microbiological Associates, Bethesda, MD (M. L. Wenk)

LAB/BRANCH

Laboratory of Comparative Carcinogenesis

SECTION

Nutrition and Metabolism Section

INSTITUTE AND LOCATION

NCI, NIH, Frederick, Maryland 21701

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.6	2.4	0.2

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The antagonisms between the essential divalent metals, calcium, magnesium, and zinc, and the divalent metal carcinogens, lead, nickel and cadmium, are under investigation in carcinogenesis, toxicity, metabolism and biochemical studies. Magnesium has been shown to inhibit lung adenoma formation in lead- and nickel-treated strain A mice as well as nickel-induced sarcomas in rats. Part of the protection afforded by magnesium against the tumorigenic activity of nickel in mice appears due to the diminished accumulation of the carcinogenic metal in the nuclei and cytosol of pulmonary cells. Nickel-induced enhancement of pulmonary DNA synthesis was abolished by magnesium. In the muscles of rats magnesium prevented the initial necrosis produced by injection of a carcinogenic dose of nickel subsulfide. Zinc deficiency caused a marked enhancement of cadmium accumulation in tissues. Excess zinc was found to markedly reduce cadmium uptake into target cells of cadmium carcinogenesis, the testicular interstitial cells. In chemical studies cadmium and nickel binding to DNA was found to be inhibited by zinc, magnesium, manganese, calcium and copper. To the extent that the biological data are available, such inhibition of DNA binding was found to be proportional to the preventive effects of the same physiological metals on cadmium and nickel carcinogenesis. The effects of calcium on lead-, cadmium-, or nickel-induced carcinogenesis are inconsistent. Extension of the mechanisms underlying the antagonistic effects of the physiologically essential divalent metals against tumor formation by nickel, lead, and cadmium in other target sites will be explored.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

L. A. Poirier	Chief, Nutrition and Metabolism Section	LCC	NCI
K. S. Kasprzak	Visiting Scientist	LCC	NCI
M. P. Waalkes	Staff Fellow	LCC	NCI
M. J. Wilson	Chemist	LCC	NCI

Objectives:

The accumulation of evidence indicates that the activated form of most organic carcinogens consists of a reactive electrophile. Possible mechanisms by which the metal carcinogens exert their activity remain relatively unexplored. The hypothesis that they act via an antagonism of the physiologically essential metals, calcium, magnesium and zinc is being tested.

Methods Employed:

The carcinogenic and toxic activities of lead, nickel and cadmium in the presence of various amounts of calcium, magnesium or zinc are studied in vivo and in vitro using standard protocols. These include long-term feeding and/or injection of the suspect compound alone, or combined with the physiological metal salt, into rats and mice followed by examination at necropsy for tumors and other pathological changes. Metabolism of the carcinogenic metals in rats and mice and in tissue culture, their effects upon thymidine incorporation into DNA, and the effects of calcium, magnesium and zinc upon the metabolism and toxicity of the carcinogenic metals are determined by radioisotopic (radiometric and autoradiographic), and standard analytical methods (atomic absorption, HPLC, amino acid analysis, chromatography, ultracentrifugation). Techniques for the isolation of viable interstitial cells of rat testes, target cells of cadmium carcinogenesis, have been developed

Major Findings:

The major contribution constituted by these studies is that the pathological effects of the carcinogenic divalent metals are very often inhibited by the physiologically essential divalent metals, magnesium and zinc. The inhibition by magnesium of nickel carcinogenesis in mice was partially the consequence of reduced nickel uptake in the target tissue (nuclei and cytosol) and of a suppression of the stimulating effects of nickel upon DNA synthesis. In rats magnesium prevented the initial necrosis caused by intramuscular injection of nickel subsulfide without affecting the gross mobilization of nickel from the injection site. It was also found that the anticarcinogenic effects of magnesium in rat muscle had a strictly local character. Magnesium was mobilized from the injection site in two days, indicating that its preventive action was accomplished during the initiation stage of carcinogenesis. The effects of calcium on nickel-induced carcinogenesis are more complex. We had found previously that calcium injections inhibited lung adenoma formation in strain A mice by lead and nickel. In the carcinogenicity trials that followed, calcium feeding or parental administration (s.c., local i.m.) had no effect on the muscle

tumor induction by i.m. nickel subsulfide in rats. However, calcium feeding enhanced the formation of renal tumors by dietary lead, while suppressing the accumulation of lead in the kidneys of rats. Zinc, which previous studies had shown to be a strong inhibitor of cadmium carcinogenesis, decreased nickel toxicity in rats by inhibiting nickel-induced hyperglycemia. Subchronic studies in rats showed that consumption of diets marginally deficient in zinc markedly increased the accumulation of cadmium in organs in which cadmium induced tumors. Treatment with 5-azacytidine, the pyrimidine analog, induced the synthesis of the hepatic zinc- and cadmium-binding protein, metallothionein, a protein which is thought to play an important role in the antagonism of cadmium carcinogenicity.

In vitro calcium, magnesium, zinc, manganese, and copper competitively antagonized the binding of cadmium and nickel to DNA, with a relative potency similar to that found in vivo for tumor prevention. Studies of the interactions of cadmium with isolated interstitial cells of testes indicate that cadmium is actively taken up by such cells and that zinc will antagonize this uptake. Pretreatment with 5-azacytidine reduced the cytotoxic effects of cadmium in cultured liver cells through an enhancement of the inducibilities of metallothionein. To date the most consistent antagonism to the pathological effects of the carcinogenic divalent metals has been provided by magnesium and zinc.

Significance to Biomedical Research and the Program of the Institute:

The aim of these studies is to increase the base of theoretical knowledge by which the potential carcinogenic hazards to man of carcinogenic metals can be diminished. Metals constitute one of the largest and broadest categories of chemical carcinogens to which humans are exposed. Metals are often among the most active carcinogens known. As a class of carcinogens they are relatively underinvestigated. The biochemical similarity of their mode of action, if any, to the organic carcinogens remains obscure. The evidence accumulated to date indicates that an antagonism to the divalent cations, calcium, magnesium or zinc, may constitute part of the mechanism by which the divalent metal carcinogens exert their activity. Successful demonstration of the molecular locus of antagonism between the physiological metals and the divalent carcinogens could help to identify the intracellular targets of metal carcinogens.

Proposed Course:

Fundamental studies on the dose-response curves, species sensitivity, route of administration and potential zinc antagonism in cadmium carcinogenesis will be conducted. Attempts will be made to extend the antagonism between carcinogenic and physiological divalent metals by determining the effects of dietary magnesium on renal carcinogenesis by lead and of zinc on sarcoma production by nickel. The possible potentiation of metal carcinogenesis by dietary deficiency of essential metals will be explored using zinc and magnesium deficiency and the cadmium and nickel tumorigenicity as a model. The intracellular targets of cadmium and nickel will be examined by determining their binding sites (e.g., metallothionein, calmodulin), functional effects and genetic damage in vivo and in vitro. Initial studies on cell transformation by nickel and cadmium will be undertaken both within the Laboratory and in collaboration with other groups.

Publications:

Kasprzak, K.S., Hoover, K.L. and Poirier, L.A.: Effects of dietary calcium acetate on lead subacetate carcinogenicity in kidneys of male Sprague-Dawley rats. Carcinogenesis 6: 279-282, 1985.

Kasprzak, K.S. and Poirier, L.A.: Effects of calcium and magnesium salts on nickel subsulfide carcinogenesis in Fischer rats. In Brown, S.S. and Sunderman, F.W., Jr. (Eds.): Progress in Nickel Toxicology. London, Blackwell, Ltd., 1985, pp. 29-32.

Kasprzak, K.S. and Poirier, L.A.: Effects of calcium and magnesium acetates on tissue distribution of carcinogenic doses of cadmium chloride in Wistar rats. Toxicology 34: 221-230, 1985.

Kasprzak, K.S., Quander, R.V. and Poirier, L.A.: Effects of calcium and magnesium salts on nickel subsulfide carcinogenicity in Fischer rats. Carcinogenesis (In Press)

Kasprzak, K.S. and Waalkes, M.P.: Role of calcium, magnesium and zinc in carcinogenesis. JNCI (In Press)

Waalkes, M.P., Kasprzak, K.S., Ohshima, M. and Poirier, L.A.: Protective effects of zinc acetate toward the toxicity of nickelous acetate in rats. Toxicology 34: 29-41, 1985.

Waalkes, M.P. and Poirier, L.A.: In vitro cadmium-DNA interactions: cooperativity of binding and competitive antagonism by calcium, magnesium and zinc. Toxicol. Appl. Pharmacol. 75: 539-546, 1984.

Waalkes, M.P. and Poirier, L.A.: Induction of hepatic metallothionein following 5-azacytidine administration. Toxicol. Appl. Pharmacol. (In Press)

Waalkes, M.P. and Poirier, L.A.: Interactions of cadmium with interstitial tissue of the rat testes: uptake of cadmium by isolated interstitial cells. Biochem. Pharmacol. (In Press)

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP04680-15 LCC

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Application of In Vitro Systems to Study Perturbations of Methyl Metabolism

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	M. J. Wilson	Chemist	LCC	NCI
Others:	L. A. Poirier	Chief, Nutrition and Metabolism Section	LCC	NCI
	U. I. Heine	Chief, Ultrastructural Studies Section	LCC	NCI
	J. L. Junker	Staff Fellow	LCC	NCI
	S. Rehm	Visiting Associate	LCC	NCI
	D. G. Blair	Chief, Microbiology Section	LMO	NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Comparative Carcinogenesis

SECTION

Nutrition and Metabolism Section

INSTITUTE AND LOCATION

NCI, NIH, Frederick, Maryland 21701

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.4	0.6	0.8

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Epithelial cells derived from the livers of 10-day-old Fischer 344 rats are used as a model system for studying the mechanism of carcinogenesis resulting from an insufficiency of methyl donors. Transformation of liver cells has been achieved following treatment with 3-deazaadenosine (DAA). This compound is metabolized to 3-deazaadenosylhomocysteine, a potent inhibitor of S-adenosylhomocysteine (AdoHcy) hydrolase, and results in an accumulation of AdoHcy, a competitive inhibitor of most physiological methylation reactions. DAA administration was shown to inhibit methylation of DNA. DNA has been isolated from tumors induced in rats initiated with DEN and fed a diet deficient in methionine and choline and used in the NIH 3T3 cell transfection assay. Results indicate that activation of an oncogene may be involved in the development of hepatocellular carcinomas in methyl-deficient rats.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliation of Professional Personnel Engaged on this Project:

M. J. Wilson	Chemist	LCC	NCI
L. A. Poirier	Chief, Nutrition and Metabolism Section	LCC	NCI
U. I. Heine	Chief, Ultrastructural Studies Section	LCC	NCI
J. L. Junker	Staff Fellow	LCC	NCI
S. Rehm	Visiting Associate	LCC	NCI
D. G. Blair	Chief, Microbiology Section	LMO	NCI

Objectives:

To determine the mechanism(s) of neoplastic transformation induced by antagonists of methyl metabolism using a cell culture model system. Specific goals are: 1) to establish the suitability of rat liver cells in culture as a model for studying the mechanism of carcinogenicity by antagonists of methyl metabolism; 2) to determine the potential carcinogenicity of known inhibitors of methylation reactions; 3) to examine the effect of these compounds on DNA methylation in order to investigate hypomethylation of DNA as a possible mechanism of transformation; and 4) to determine the transforming potential, by transfection into NIH 3T3 cells, of DNA isolated from hepatocellular carcinomas induced by methyl-deprivation and to determine the oncogene(s) responsible for such transformation as well as the mechanism of its activation.

Methods Employed:

Rat liver cells (TRL 1215) were treated with DAA for up to twelve weeks then maintained in culture in the absence of DAA. Tumorigenicity and anchorage-independent growth were monitored at monthly intervals. The percentage of cytosine residues modified to 5-methylcytosine are determined in TRL 1215 cells undergoing treatment with DAA at doses known to induce transformation. DNA is isolated, purified, subjected to enzymatic hydrolysis and the hydrolysate chromatographed on HPLC. DNA is isolated from hepatocellular carcinomas produced in F344 rats following prolonged dietary deprivation of methyl donors with and without prior initiation with DEN (20 mg/kg body weight). The average molecular weight of isolated DNAs, determined by agarose gel electrophoresis, is greater than 50 kilobases. DNA transfections of NIH 3T3 cells are performed as described (Cooper, et al., Cancer Res., 44: 1-10, 1984). The cultures are scored for morphologically transformed foci after 23 days. High molecular weight DNA is isolated from primary transformants, used in a second cycle of transfection and secondary transformants isolated.

Major Findings:

A low tumor incidence was observed in animals injected with cells treated for up to twelve weeks with 0.075 or 0.100 mM deazaadenosine. Tumor incidence increased when cells were maintained in culture for an additional one or two months in the absence

of deazaadenosine. Growth in soft agar was also observed at these time points. Tumors were not present in animals injected with cells treated with 0.150 mM deazaadenosine for the experimental period described. However, this experiment is still in progress, and we have detected tumors in animals injected with cells maintained in culture for longer periods of time. We think that the delay in these cells becoming tumorigenic may be due to the lingering toxic effects of deazaadenosine observed at this dose level. The control cells have remained negative throughout this experiment. The level of DNA methylation in cells treated with 0.075 mM deazaadenosine for one month was 38% less than that of untreated cells. A similar level of suppression of methylation was observed in cells treated for three months. DNA of cells treated for three months then maintained in the absence of deazaadenosine for one month, as was done in the transformation study, was somewhat less hypomethylated. Cells treated for three months then cultured an additional three months had methylation levels identical to control values.

High molecular weight DNA isolated from liver tumors induced in rats by DEN initiation followed by methyl-deprivation has produced transformed foci in the NIH 3T3 cell transfection assay. Approximately 20% of tumor DNAs tested were positive while all control DNAs have been negative. The presence of rat DNA in the primary transformants thus far examined has been demonstrated using a probe specific for rat repetitive sequences (gift of Dr. A. Furano, NIH). The data obtained indicate the presence of an activated oncogene in the DNA of hepatocellular carcinomas from rats resulting from the exposure to a carcinogenic regimen.

Significance to Biomedical Research and the Program of the Institute:

The role of methyl donors in tumor development is an intriguing area of carcinogenesis research. Finding the answers to fundamental mechanistic questions of neoplastic transformation resulting from perturbation of methyl metabolism may best be approached, at least in part, in vitro rather than in vivo. The use of a cell line derived from the liver, the target tissue of methyl insufficiency, obviously is of great benefit. It is also possible, using liver cells in culture, to test the transforming potential of compounds which would pose problems of stability and expense in in vivo test systems.

Transformation of NIH 3T3 cells with DNAs from tumors induced by methyl deprivation is significant for two reasons. First, the determination of the mechanism whereby the transforming oncogene is activated would provide important information regarding the mechanism of methyl deficiency carcinogenesis. Second, it would demonstrate that oncogene activation also occurs in the liver, an observation not previously reported.

Proposed Course:

Rat liver cells in culture will continue to be used as a model system for investigating the mechanism of neoplastic transformation resulting from the perturbation of methyl metabolism. Specifically, the uptake of AdoEt into cells in culture will be examined using cycloleucine to inhibit biosynthesis of AdoEt. It is important to determine and to compare the intracellular concentrations of AdoEt resulting from the exposure of cells to equimolar concentrations of AdoEt and ethionine. In addition, the effect of both compounds on DNA methylation will be assessed. This is a

particularly important experiment for furthering the understanding of the relevance of DNA methylation to the mechanism of ethionine-induced carcinogenesis. Other antagonists of methyl metabolism, including azacytidine and polyamines, will be tested for their ability to transform cells and to induce hypomethylation of DNA. The purpose of these studies will be to determine if the observed correlation between transformation and DNA hypomethylation can be extended to other compounds. The use of serum-free medium (developed by Dr. Thomas Iype, FCRF) will make it possible to determine if the neoplastic potential of methionine and/or choline deprivation observed in vivo can be extended to cells in culture.

The transfection studies currently in progress will be continued. In collaboration with Dr. Donald Blair of the Laboratory of Molecular Oncology, identification of the oncogene activated in methyl-deficient tumors will be pursued as will its mechanism of activation. In addition, DNA from rat liver cells transformed in culture by ethionine and DAA will be examined for the presence of active oncogenes. Messenger RNA from cells frozen at various stages of the DAA transformation study will be isolated. These mRNAs will be used in hybridization studies with probes of known oncogenes to determine if there is a sequential expression of oncogene(s) during transformation. These hybridization experiments will also be performed on mRNA obtained from liver tumors induced by methyl deprivation as well as on mRNA obtained from cell lines which have been initiated from these tumors and their metastases.

Publications:

Junker, J.L., Cottler-Fox, M., Wilson, M.J., Munoz, E.F. and Heine, U.I.: Transformation associated increase of adhesion, cellular fibronectin and stress fiber development in a liver epithelial cell line. JNCI 74: 173-183, 1985.

Junker, J.L. and Wilson, M.J.: Divergent expression of laminin and fibronectin in nontumorigenic and transformed liver epithelial cells. J. Cell Sci. (In Press)

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP04812-17 LCC

PERIOD COVERED
October 1, 1984 to September 30, 1985

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)
Cell Interactions During Transformation of Epithelial Cells

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	U. I. Heine	Chief, Ultrastructural Studies Section	LCC	NCI
Others:	J. L. Junker	Staff Fellow	LCC	NCI
	M. J. Wilson	Chemist	LCC	NCI
	K. Takahashi	Visiting Associate	LCC	NCI
	C. H. Fox	Sr. Scientist	LC	NCI

COOPERATING UNITS (if any)

Biological Products Laboratory, Program Resources, Inc., FCRF, Frederick, MD
(E. F. Munoz)

LAB/BRANCH
Laboratory of Comparative Carcinogenesis

SECTION
Ultrastructural Studies Section

INSTITUTE AND LOCATION
NCI, NIH, Frederick, Maryland 21701

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.5	2.0	0.5

CHECK APPROPRIATE BOX(ES)
☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

In order to characterize the changes in cytoskeletal, cell-cell and cell-matrix interactions which are indicative of epithelial cell transformation, we have used as a model system ethionine-transformed liver epithelial cells of the TRL 1215 cell line for which controls are available both at low and at high passage levels. In this line transformation is accompanied by an increase in cell-substrate adhesion, cell spreading, marked by increased amounts of actin stress fibers, vinculin plaques, adhesion plaques, and cell-associated fibronectin. Thus, the capacities for tumorigenicity and anchorage independent growth coexist with the capacity for a high level of cell-substrate adhesion in these cells. At the same time there are decreases in the immunocytochemical expression of laminin and in the extent of junctional complexes, indicating that changes in the specific molecular interactions involved in cell-cell and cell-substrate adhesion are more important to transformation than changes in adhesion per se. The finding of increased fibronectin in tumor-bearing livers of ethionine-fed rats, by paralleling the results obtained in vitro, shows the value of the TRL 1215 line as a model which relates to in vivo carcinogenesis. The promotable mouse epidermal cell line, JB6, has been subjected to a similar protocol to define promotion specific events. To establish a phenotypically transformed, tumorigenic cell type, a combination of repeated exposure to the promoter and clonal propagation of anchorage independent cells is necessary. Our novel finding regarding the presence of the H-ras gene product, p21, in focus-forming cells of line JB6 may be indicative of the protein's role in initiating focus formation in addition to its known role in cell proliferation.

PHS 6040 (Rev. 1/84)

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

Ursula I. Heine	Chief, Ultrastructural Studies Section	LCC	NCI
James L. Junker	Staff Fellow	LCC	NCI
Mary J. Wilson	Chemist	LCC	NCI
Kiyoshi Takahashi	Visiting Associate	LCC	NCI
Cecil H. Fox	Sr. Scientist	LB	NCI

Objectives:

To determine the morphological correlates of promotion and transformation. Particularly, to characterize changes in cytoskeletal, cell-cell and cell-matrix interactions which are indicative of epithelial cell transformation, using as a model system control and ethionine-treated liver epithelial cells. To examine the role of the cytoskeleton and cytomatrix in cell behavior. To compare results from cultured cells to results from tumor-bearing tissue in order to evaluate the relationship of the in vitro model to tumor development in vivo. To determine, by expanding the study to include other cell lines, markers of tumorigenic transformation as well as of tumor promotion among epithelial cell lines in general. To examine the role of the H-ras oncogene in tumor promotion.

Methods Employed:

Two cell lines, TRL 1215 rat liver epithelial cells (Idoine, J.B. et al. In Vitro 12: 541-553, 1976) and JB6 (N. Colburn, Project Number Z01CE05383-01 LVC) and their appropriate controls, were the model systems in our studies. Three sublines were examined in the liver cell system: ethionine-treated cells transformed following exposure to 7.5 mM DL-ethionine in the culture medium and untreated cells at low and high passage levels. Tumor promotion in JB6 cells was accomplished by repeated exposure of initiated cells to the tumor promoter 12-O-tetradecanoylphorbol-13-acetate (TPA). Progression of cells to the transformed phenotype was achieved by combining repeated treatment with TPA and clonal propagation. Transformation was measured by colony formation in soft agar and by tumor production in vivo. Routine cell culture methods were used for the propagation of the cell lines. The distribution of cytoskeletal proteins (tubulin, keratin, vimentin, actin, vinculin), extracellular matrix components (fibronectin, laminin), and the H-ras oncogene product, pP21, was determined by indirect immunofluorescence. Variations in cell spreading on the substratum were determined by morphometric analysis of projected cell area. Adhesion structures were examined by reflection contrast microscopy. Scanning, transmission and immunoelectron microscopy were used for the high resolution examination of cell structure and cell-cell adhesion.

Major Findings:

It has been established that the TRL 1215 cells are epithelial cells in which, in contrast to the stereotypical transformed phenotype, transformation is accompanied by an increase in cell-substrate adhesion as indicated by increased numbers of actin

stress fibers, fibronectin fibers and adhesion plaques in ethionine-transformed cells. Yet, these transformed cells form multilayered foci and are fully capable of anchorage independent growth and of producing tumors in syngeneic animals. Vinculin plaques are also found to be increased, as would be expected from vinculin's localization at adhesion plaques and other locations where actin filaments insert into the plasmalemma. Staining for laminin, however, decreases with transformation when areas of flattened polygonal cells are compared. Thus, the two matrix components fibronectin and laminin have a divergent immunocytochemical expression in this cell line. The observed decrease in laminin is not caused by an inability of the transformed cells to synthesize the protein, since laminin is deposited in multilayered areas. Laminin staining in low passage control cells is observed to encircle individual cells. In addition, intermediate junctions are more extensive in this subline, and vinculin and actin staining is concentrated at the edge of the cell in regions of cell to cell contact.

In studies of growth on defined substrata, all three sublines show a much greater degree of cell spreading on Type IV collagen and fibronectin than on laminin, bovine serum albumin, or uncoated plastic. However, the data so far do not indicate that any of the substrata affect the growth rate.

In vivo studies were begun by an immunofluorescence examination of the fibronectin distribution in livers of rats fed a diet containing ethionine. In direct correlation in the in vitro studies, fibronectin was increased in tumor-bearing livers, and was found surrounding hypertrophied tumor cells.

Exposure to TPA of promotable (P+) cells and nonpromotable (P-) cells of JB6 cultures results in loss of actin stress fibers, fibronectin mat and vinculin plaques. In the presence of TPA the effect on the cytoskeleton of the P- cells is only transient, and these cells have been found capable of reverting to their original phenotype; however, such repair mechanisms appear to be nonexistent in P+ cells. P+ cells progress to form foci; yet, upon return to standard culture conditions, focus formation and phenotypic changes are reversed. The H-ras oncogene product, p21, is expressed only in mitotic and focus-forming cells, indicating a specific function of this protein in focus formation and loss of contact inhibition in addition to its known role in cell proliferation. To obtain P+ cells with an irreversibly transformed phenotype, repeated exposure to TPA combined with clonal propagation is necessary. Cells of such cultures are tumorigenic upon injection into nude mice.

Significance to Biomedical Research and the Program of the Institute:

Cell-cell and cell-substrate adhesion are critical factors in the regulation of growth and development. Thus, in order to understand malignant conversion and the mechanisms of unrestrained growth, invasion, and metastasis, it is necessary to determine how a cell's interactions with surrounding cells and matrix are altered by transformation. It is important that these determinations be made in epithelial cell systems since the great majority of human cancers are carcinomas. The findings from the TRL 1215 liver epithelial cell system, in which transformation is accompanied by increased cell-substrate adhesion, show that it is not necessary for a cell to lose this ability in order to be transformed. The divergent immunocytochemical expression of fibronectin and laminin, in which only fibronectin expression correlated with maximal cell spreading and adhesion, provides further evidence of a direct role for fibronectin in adhesion. The shift in the composition of the extracellular matrix

from laminin to fibronectin during transformation relates to the in vivo finding that laminin-containing basement membranes are disrupted in malignant but not in benign tumors, and indicates that changes in the specific molecular interactions of adhesion are more important in transformation than changes in adhesion per se. The corresponding reduction in junctional complexes suggests a supportive role for laminin in the formation or stabilization of cell-cell contacts that is lost during transformation. The finding of increased fibronectin in livers of ethionine-fed rats, by paralleling the results in vitro, shows the value of the TRL 1215 line as a model of in vivo carcinogenesis.

Tumor promotion may play an important role in determining the outcome of human exposure to environmental carcinogens. Previous experiments favor a nonnuclear site as specific target for phorbol esters, i.e., cytoskeleton-membrane interactions. Our studies, however, indicate for the first time the production of a transforming protein in response to a tumor promoter, and suggest that such events may occur in vivo during tumor promotion. As we have shown, only promotable cells lose contact inhibition after confluence and produce foci in the presence of TPA; H-ras p21 is exclusively expressed in cells of such foci. As removal of TPA permits the cells to revert to normal appearance with concurrent loss of ras-oncogene expression, we conclude that the altered phenotype in focus producing cells may be caused directly by the TPA-dependent, continuous production of the ras-oncogene product.

Proposed Course:

The differences between transformed and nontransformed cells in their cell-substrate and cell-cell interactions will continue to be examined in TRL 1215 cells, and these investigations will be extended to other epithelial cell systems.

Studies on the effects of known basement membrane components, fibronectin, laminin, and Type IV collagen, on the structure and growth of TRL 1215 cells will be continued using cells grown in culture dishes onto which these substances have been absorbed. Cell spreading will be measured morphometrically, adhesion structures examined by reflection-contrast microscopy, and growth rates determined. Results using routine culture medium and fibronectin-depleted culture medium will be compared. In order to identify other components of the cytomatrix that may play a significant role in the altered behavior of transformed cells, further experiments will include an analysis of surface adhered material to determine if there are minor or unknown matrix proteins which vary markedly in transformed and nontransformed cells.

Investigations of cell-cell interactions will focus on the nature and distribution of different types of cell junctions. To define the nature of the intermediate junctions and to examine the relationship between laminin and the junctional complexes, immunoelectron microscopy of junctional areas, using antilaminin, antivinculin, and, as an actin probe, heavy meromyosin, will be done. Tight junctions and gap junctions will also be studied by examining freeze-fracture replicas.

The relationship between in vitro and in vivo ethionine-induced transformation will be studied by continuing light microscopic immunocytochemistry, focusing on identifying the specific antibody staining patterns of the extracellular matrix of tumor tissue.

The question arises as to whether observed TPA-dependent phenotypic transformation and the activation of ras-oncogene expression are common elements of tumor promotion in epithelial cells. To clarify this point, it will be determined if other promoters, such as benzoyl peroxide (BP), provoke changes similar to TPA. This is of special interest as the mode of action of BP is completely different from TPA. Furthermore, we hope to test the effects of promoter antagonists on TPA-induced transformation. Inhibitors of tumor promotion have been useful in the identification of important events in tumor promotion. Thus, the study of their action may be especially helpful in clarifying the role of the ras-oncogene activation. We plan to use in this study stage 1 and stage 2 blockage agents, after having initiated appropriate experiments to identify these stages in our model system.

Publications:

Junker, J.L., Cottler-Fox, M., Wilson, M.J., Munoz, E.F. and Heine, U.I.: Transformation-associated increase of adhesion, cellular fibronectin, and stress fiber development in a liver epithelial cell line. JNCI 74: 173-183, 1985.

Junker, J.L. and Wilson, M.J.: Divergent expression of laminin and fibronectin in nontumorigenic and transformed liver epithelial cells. J. Cell Sci. (In Press).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05092-07 LCC

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Transplacental Carcinogenesis and Tumor Promotion in Nonhuman Primates

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	A. E. Palmer	Research Veterinarian	LCC	NCI
Others:	J. M. Rice	Chief	LCC	NCI
	J. M. Ward	Chief, Tumor Pathol. and Pathogen. Section	LCC	NCI
	L. M. Anderson	Expert	LCC	NCI
	P. J. Donovan	Chemist	LCC	NCI
	A. O. Perantoni	Microbiologist	LCC	NCI

COOPERATING UNITS (if any)

Meloy Laboratories, Inc., Rockville, MD (Dr. J. Phillips and S. Harbaugh)

LAB/BRANCH

Laboratory of Comparative Carcinogenesis

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Frederick, Maryland 21701

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
3.2	2.0	1.2

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Nonhuman primates of the species Erythrocebus patas (patas), Macaca mulatta (rhesus), Macaca fascicularis (cynomolgus) and Cebus apella (cebus), are subjected to direct-acting or metabolism-dependent chemical carcinogens by transplacental or direct exposure. In some cases the carcinogen-treated animals are subsequently exposed to chemicals that promote the development of neoplasms in rodents. Mechanisms of organ and species differences in the effects of chemical carcinogens and tumor promoters among rodent and nonhuman primate species are investigated. DNA repair capacity and its inducibility in different tissues are being surveyed in this context. Induced tumors are evaluated by light microscopy using standard staining procedures, histochemical techniques and electron microscopy and are assayed for in vitro cultivability and transplantability to rodents. Selected tumors are subjected to DNA extraction and attempts are made to transfect NIH 3T3 cells with their DNA.

PHS 6040 (Rev. 1/84)

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

A. E. Palmer	Research Veterinarian	LCC	NCI
J. M. Rice	Chief	LCC	NCI
J. M. Ward	Chief, Tumor Pathology and Pathogenesis Section	LCC	NCI
L. M. Anderson	Expert	LCC	NCI
P. J. Donovan	Chemist	LCC	NCI
A. O. Perantoni	Microbiologist	LCC	NCI

Objectives:

To study and characterize the variable sensitivities of different organ systems in nonhuman primates to carcinogens which act directly or require in vivo metabolism for carcinogenic activity during the prenatal and postnatal periods. To precisely characterize neoplastic and selected nonneoplastic lesions from treated animals by light and electron microscopy, histochemistry, explantation to cell or organ culture, transplantation and by other procedures which may be appropriate. To attempt to demonstrate the phenomenon of tumor promotion in nonhuman primates and to determine whether cell and tissue specificities of tumor-promoting chemicals demonstrated in rodents are similar in nonhuman primate models.

Methods Employed:

Carcinogenic chemicals specifically selected or designed for a given study are purified after purchase or synthesized de novo and characterized thoroughly by chromatographic and spectroscopic procedures. Radiolabeled compounds are similarly prepared as required. The carcinogens are administered in precise doses to nonpregnant or exactly timed-pregnant nonhuman primates (patas, rhesus, or cynomolgus monkeys) and the treated animals and their offspring are followed carefully for the development of tumors. Agents which previously have been demonstrated to promote tumors in rodents are similarly purchased, purified or synthesized de novo and administered to primates after completion of an initiating regimen of exposure to organ-specific carcinogens.

Tumor-bearing nonhuman primates are intensively monitored to study tumor growth, body weight and clinical pathological changes. Selected animals are evaluated for tumor markers such as alphafetoprotein. Selected tumors which may cause suffering are carefully evaluated on an individual basis and may be surgically removed to reduce suffering and to prolong life. When tumors are judged to be inoperable and to be causing suffering or threatening life, animals are killed by euthanasia. Thorough gross postmortem examinations are performed and all gross lesions plus sections from all major organs are evaluated by light microscopy. Selected neoplasms are evaluated by electron microscopy and/or are cultured by cell or explant culture in vitro, then transplanted to athymic (nu/nu) mice. Selected tumors are subjected to DNA extraction and attempts are made to transfect NIH 3T3 cells with their DNA.

Major Findings:

Studies on carcinogenesis by ethylnitrosourea (ENU) and diethylnitrosamine (DEN) in Erythrocebus patas, an Old World monkey, continue and have been expanded to include aflatoxin B_1 (AFB_1). Additional cases of mesenchymal and epithelial tumors were observed in the offspring of monkeys that received ENU intravenously during pregnancy, especially when exposure occurred during the first trimester of gestation. These additional findings support the tentative conclusions drawn previously that, like rodents, this species of nonhuman primate is quantitatively more susceptible to the direct-acting alkylating agent, ENU, during prenatal life, with animals exposed in utero exhibiting a higher incidence of tumors after a shorter latency than juvenile or adult animals that received a similar dose directly. Neoplasms continue to develop in primates now as old as 126 months (middle aged adults) that were exposed to low doses of ENU transplacentally during the first trimester of gestation (30 to 60 days).

Tumors induced in transplacentally exposed patas monkey include both embryonal and adult cellular types. Embryonal tumors have occurred at low frequency and have included nephroblastomas and rapidly-growing, lethal primary sarcomas of the lung. Patas monkeys treated transplacentally which survive to adulthood and those exposed directly to ENU have developed multiple bronchioalveolar adenomas of the lung, suggesting a biphasic response of this monkey's lungs to this carcinogen. Small cell carcinomas originating in the lungs have been observed in patas and rhesus monkeys treated transplacentally with ENU.

A total of nine female patas monkeys have died from a widely disseminated, hemorrhagic malignancy less than seven months after the termination of a pregnancy during which they received intravenous ENU. The tumor has been characterized by small primary uterine lesions with metastatic lesions, primarily to the lungs. Morphologically the tumor cells in these cases resemble cytotrophoblast, and are rapid-growing, causing death by hemorrhage. Tumor cells were found within placenta in one case and were usually widely disseminated within uterine and pulmonary vessels. One animal developed a relatively slow growing tumor of the uterus which invaded the pelvic organs. This tumor was predominantly of cytotrophoblast type, but also contained syncytiotrophoblast-like cells. Attempts to identify chorionic gonadotropins by serological means, using antisera that are reactive with the chorionic gonadotropins of man, the great apes, macaque monkeys, and several New World monkeys, were unsuccessful. However, biological activity has been identified in sera from pregnant patas monkeys using the mouse testicular interstitial cells in vitro. This activity occurs only between days 19 and 34 of gestation. Sera collected from five females with the rapidly fatal tumor at 0 to 47 days antemortem were tested by this biological test and all were negative. However, the serum from the animal with the tumor containing syncytiotrophoblast contained significant activity beginning 167 days antemortem, and the activity levels increased until the time of her death.

These observations provide the first chemically inducible animal model for gestational choriocarcinoma and suggest that chemicals may play a role in the development of this malignancy in humans, since incidence differs markedly throughout the world. Incidence in the human disease is highest in southeastern Asia where the chance of exposure to naturally occurring carcinogens, such as aflatoxin F_1 (AFB_1), is high. For this reason, studies are underway to investigate the carcinogenic activity of AFB_1 in the pregnant patas monkey.

In studies to demonstrate the phenomenon of tumor promotion in nonhuman primates, the liver is being used as the target organ. DEN, the most effective chemical carcinogen in nonhuman primates is used as an initiator, and to date, the barbiturates, PB and sodium barbital, are under study as promoters. Patas monkeys treated with DEN by the intravenous, transplacental and intraperitoneal routes were later subjected to daily doses of 15 mg/kg of PB in their drinking water. Tumor promotion occurred only in the liver and was evident regardless of the route of DEN exposure and despite an interval of approximately 48 months after DEN given intravenously and transplacentally before the PB was started. Studies are underway, using the cynomolgus monkey (Macaca fascicularis), to investigate possible promoter activity by PB and sodium barbital.

Liver cytochrome P-450 and aminopyrine demethylase were measured in patas monkeys which received either DEN only or DEN plus 275 days of PB at 15 mg/kg/day. Levels of cytochrome P-450 were 3.1-fold and of aminopyrine dimethylase 3.6-fold higher in the PB recipients.

Significance to Biomedical Research and the Program of the Institute:

Research on animal models of human childhood neoplasms should provide an insight into the types of causative agents and modes of exposures responsible for childhood cancer cancer. It is to be expected that natural selection would tend to eliminate genotypes in the human population which predispose individuals to the development of fetal neoplasms before attaining reproductive age, yet the incidence of embryonal neoplasia in childhood is relatively constant. Epidemiological studies have pointed to the occurrence of childhood neoplasms in association with specific chromosomal deletions and rearrangements and with certain types of congenital malformations which are not inherited. This suggests that environmental agents, alone or in combination, may play a role in the induction of such neoplasms. The inducibility of tumors very similar to the pediatric tumors of man by chemical carcinogens in laboratory rodents and primates further supports this view. Most tumors induced transplacentally in rodents are of adult types and appear during adult life in individuals exposed in utero, resembling the human experience with diethylstilbestrol. The ENU studies in monkeys have provided experimental data indicating that both adult and pediatric tumor types develop in two species of primates in response to carcinogenic exposure in utero and suggest that chemical carcinogens may be involved in the prenatal genesis of pediatric and possibly certain adult types of tumors in man. The demonstration of the inducibility of uterine choriocarcinoma by chemical carcinogens further illustrates the importance of preventing human exposure to carcinogenic chemicals during pregnancy in either the workplace or environment.

The phenomenon of tumor promotion, while well established in rodents, is based on very limited data from which to extrapolate to man. The generality of the phenomenon and the extent to which organ-specific effects can be predicted in one species on the basis of bioassays conducted in another remain to be established. For tumor promotion, there is, as yet, no unifying conceptual hypothesis exploitable for interspecies comparison comparable to the role of primary damage to DNA in mutagenesis and probably in neoplastic transformation by genotoxic chemicals. It appears from experiments in rodents that promotion, unlike tumor initiation, is not persistent and that the underlying toxic effects thus are not cumulative. If, as seems likely, tumor promotion plays a significant role in the development of human cancer, the requirement for continual exposure to the promoting agent may provide prevention

strategies that are much more readily applicable than in the case of persistent and cumulative genetic toxicity.

Proposed Course:

Monkeys exposed to carcinogens and/or promoter compounds will be observed for abnormalities related to these treatments. These observations include gross palpation, serum chemistry evaluations, and testing for selected serum tumor markers, e.g., alphafetoprotein and carcinoembryonic antigen. Laparotomies and biopsies of selected organs or tumors of animals under study will utilize histological, histochemical, immunohistochemical and tissue enzyme levels, attempting to follow the progression of tumors during the carcinogenic process.

The formation of DNA adducts and their persistence in the tissues of carcinogen-treated nonhuman primates will be studied by immunological methods as reagents become available for such studies.

The identification of the period of chorionic gonadotropin secretion in pregnant patas monkeys will permit the collection and purification of the patas chorionic gonadotropin. Once isolated, attempts will be made to develop monoclonal antibodies against this hormone and to use these antibodies to study a) the hormone secretion pattern in pregnant patas monkeys and b) the hormone secretion by gestational choriocarcinoma induced in this species.

Publication:

Rice, J.M.: Exposure to chemical carcinogens during pregnancy: consequences for mother and conceptus. In Patillo, R.A. and Hussa, R.O. (Eds.): Human Trophoblast Neoplasms. New York, Plenum Publishing Corp., 1984, pp. 179-202.

Winterer, J., Palmer, A.E., Cicmanec, J., Davis, E., Harbaugh, S. and Loriaux, D.L.: Endocrine profile of pregnancy in the patas monkey (Erythrocebus patas). Endocrinology 116(3): 1090-1093, 1985.

CONTRACTS IN SUPPORT OF THIS PROJECT

MELOY LABORATORIES, INC. (N01-CP-41016)

Title: Resources for Transplacental Carcinogenesis in Primates

Current Annual Level: $274,866

Man Years: 3.2

Objectives:

This project is designed to demonstrate and characterize transplacental carcinogenesis in nonhuman primates, especially the Erythrocebus patas, an Old World monkey. Additionally, related phenomena are studied, including the increased risk of carcinogenesis in adult females exposed to chemicals during pregnancy, tumor promotion, and mechanisms of cell and organ specificities and of species differences in the effects of both chemical carcinogens and tumor promoters.

Major Contributions:

Ethylnitrosourea (ENU) has been shown to be a potent carcinogen in the rhesus (Macaca mulatta) and patas monkeys. In both species the fetus is more susceptible than is the adult, and this susceptibility is more pronounced during the first and early second trimesters of pregnancy. However, the kinds of tumors seen in the two species differ in their characteristics and distribution.

Diethylnitrosamine (DEN) given to pregnant patas monkeys during gestation did not cause tumors in the offspring or mothers after four years of observation. However, after 24 to 30 months of subsequent daily doses of phenobarbital comparable to therapeutic anticonvulsant levels in man, both offspring and mothers developed hepatocellular adenomas and carcinomas. Phenobarbital clearly can promote hepatocarcinogenesis in this species as it does in rats.

Except for the association between in utero exposure to diethylstilbestrol and the increased risk of vaginal adenocarcinoma during early adulthood, there is little known concerning the effects of carcinogens on the human fetus. Transplacental chemical carcinogenesis studies have been limited to rodent species which differ greatly from man. Most significant is the more rapid rate of fetal and neonatal growth and maturation in rodents. Nonhuman primates also have shorter gestations and mature more rapidly than do humans, but they are more similar to man in fetal growth, placentation and early development than are rodents. Tumors induced to date in rhesus and patas monkeys by transplacental exposure to carcinogens resemble some congenital tumors or tumors of infancy and childhood seen in man, suggesting that prenatal exposure of humans to chemicals may be a factor in tumor incidence. The demonstration of tumor promotion in nonhuman primates provides significant evidence of the importance of this phenomenon to man.

Proposed Course:

Animals previously exposed to carcinogens will continue to be closely monitored for tumor development, and all tumors will be intensively studied. In addition, studies to demonstrate more precisely the varying sensitivity of the fetus during gestation are under way. Limited numbers of animals will be treated with agents known to be promotive in rodents, after limited transplacental exposure to carcinogens. The transplacental effects of chemicals other than ENU and DEN will be explored. The direct and transplacental carcinogenic effects of aflatoxin B_1 will be studied.

MELOY LABORATORIES, INC. (N01-CP-25613)

Title: Tumor Promotion in Cynomolgus Monkeys (*Macaca fascicularis*)

Current Annual Level: $116,793

Man Years: 1.5

Objectives:

This project is intended to demonstrate the phenomenon of tumor promotion in cynomolgus monkeys and to explore the promotive activity in this species of several chemicals known to promote tumors in rodents. The liver model was chosen because diethylnitrosamine (DEN) has been studied extensively and shown to be a predictable hepatocarcinogen in this species. Preliminary findings suggest that DEN initiates patas monkey liver when given intravenously or transplacentally. The effect of promoter compounds in vivo on liver metabolism, morphology and enzyme induction will be studied.

Major Contributions:

The contract is in its third year and preliminary studies have shown major differences in the in vivo response of cynomolgus liver as compared to Fischer rat liver to several chemicals known to promote liver tumors in rats.

A major portion of the colony is under study for DEN initiation followed by promotion with promoter compounds. Animals are being closely monitored for tumor development, both by gross examinations and serum alphafetoprotein levels. To date no tumors have been identified.

Proposed Course:

Study animals will be closely monitored for evidence of tumor development. Tumors may be surgically excised in selected cases to determine the effect of this procedure on developing tumors and to study the pathogenesis of the process. Whenever tumor-load is determined to be causing suffering or is life threatening, the animal will be killed by euthanasia. Tumors will be studied histologically and histochemically by light and electron microscopy, by cell or organ culture for growth characteristics and by transplantation into athymic mice. Selected tumors will be studied by DNA transfection and hybridization to determine the presence of and/or expression

will be studied by DNA transfection and hybridization to determine the presence of and/or expression of oncogenes. Studies to determine the impact of chemical liver tumor promoters on the induction of liver enzymes in the cynomolgus monkey liver and to compare this behavior both to that of nonpromoters and to the effect of these chemicals on rodent liver are planned.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05093-07 LCC

PERIOD COVERED
October 1, 1984 to September 30, 1985

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)
In Vitro Studies on Organ Specificity in Transplacental Carcinogenesis

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	J. M. Rice	Chief	LCC	NCI
Others:	P. J. Donovan	Chemist	LCC	NCI
	A. O. Perantoni	Microbiologist	LCC	NCI
	O. Barbieri	Guest Researcher	LCC	NCI

COOPERATING UNITS (if any)

Microbiological Associates, Inc., Bethesda, MD (M. L. Wenk)

LAB/BRANCH
Laboratory of Comparative Carcinogenesis

SECTION
Perinatal Carcinogenesis Section

INSTITUTE AND LOCATION
NCI, NIH, Frederick, MD 21701

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
3.2	2.2	1.0

CHECK APPROPRIATE BOX(ES)
☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The roles of morphogenetic differentiation in controlling the phenotypic expression of neoplastic transformation, the degree of malignancy of tumors, and the susceptibility of developing organs to carcinogenesis are studied using organ culture and tissue transplantation techniques, with current emphasis on the kidney. A defined medium for growth of rat and mouse ureteric bud epithelium in monolayer culture has been developed in which epidermal growth factor and selenium have proved essential and insulin, hydrocortisone, and transferrin have proved highly beneficial. The ability of transplacentally administered carcinogens to induce genotoxic damage in cells of embryos or fetuses exposed at different stages of gestation was determined for rat, mouse, and Syrian hamster. Cells were isolated from exposed embryos and gene mutations at two to three loci (resistance to ouabain and 6-thioguanine, and to diphtheria toxin in the hamster) were assayed in vitro with simultaneous determination of survival ability. Organ specificity of induced gene mutation is being determined in embryonal cells isolated from organs of various species exposed in utero at comparable stages of gestation. A maximum level of mutation induction was found to be inducible by N-nitrosoethylurea at day 9 of gestation for mesenchymal cells of the Syrian hamster and at day 9-10 for the F344 rat.

PHS 6040 (Rev. 1/84)

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel engaged on this Project:

J. M. Rice	Chief	LCC	NCI
P. J. Donovan	Chemist	LCC	NCI
A. O. Perantoni	Microbiologist	LCC	NCI
O. Barbieri	Guest Researcher	LCC	NCI

Objectives:

To identify and characterize those aspects of morphogenetic differentiation which modify the consequences of prenatal exposure to chemical carcinogens, especially in the nervous and genitourinary systems. The ultimate objective is to elucidate the control of expression of the neoplastic phenotype in transformed cells. To devise and apply improved quantitative selective mutation systems to embryonal and fetal primary cells in culture, from donors previously treated in utero with chemical carcinogens. To determine the time course of maximum sensitivity to induced gene mutation of cells from embryos or fetuses transplacentally exposed to carcinogens at different stages of gestation. To determine quantitative dose curves for transplacentally induced gene mutation by selected carcinogens. To determine the sensitivity of various species to metabolism-independent transplacental chemical carcinogens and to determine inter- and intra-litter variations in response. To determine the organ specificity in various species of gene mutation transplacentally induced by nitrosoethylurea. To apply in vitro transformation assays to cells isolated from embryos of different species treated transplacentally with chemical carcinogens. To correlate the above in vitro quantitatively determined parameters with transplacental tumorigenesis data.

Methods Employed:

Direct mutagenicity assay. Gravid animals are injected with different doses of agents at precise periods of gestation. Primaries are made from either the whole fetus or selected organs of different species. Cells are cultured for an experimentally determined expression time and either conventional or selective media. Cells are then seeded for determination of survival and treated with the selective agent including diphtheria toxin, 6-thioguanine or ouabain. Cells are either grown in conventional media (Dulbecco's MEM) 10% fetal bovine serum for the Syrian hamster, 15% fetal bovine serum for the rat, or 10% horse serum for the mouse. Specific cell types are grown. Brain derived cells or putative astrocytes are grown according to the method of Sellbrenner in 10% fetal calf serum. Keratinocytes are grown in MCDB 153 with low calcium serum in the same concentrations for the different species as before. Lung cells are grown in MCDB 153 formulation of Peale and Ham which is a serum-free medium. Growth of hamster liver was negative under all conditions and therefore we are now attempting to culture rat and mouse hepatocytes using different methodologies.

Renal differentiation. Short- and long-term cell and organ culture techniques are developed and the features of tissue rudiments maintained therein characterized

by histochemical, light microscopic, and ultrastructural techniques. Cultures of both normal fetal organ rudiments and selected tumors are utilized to explore the effects of morphogenetic differentiation and its induction on the behavior of tumors of undifferentiated cell type, including nephroblastic tumors of the kidney. Tumors are induced transplacentally or by direct treatment in experimental animals to provide suitable material for study and are transplanted serially in appropriate recipients to develop standard, manipulable models for studies in vitro. Surgical procedures relating to tissue transplantation are adapted as necessary to study the capacity of various recipient sites to modify differentiation of selected transplantable tumors. Substances and tissues known to influence differentiation are combined with tumors and normal undifferentiated inducing tissues, and interactive events in the differentiation of normal and neoplastic tissues are characterized. All studies are performed in more than one species, selecting species (such as the rat and mouse) which can be readily manipulated in the laboratory and in which responses of selected organ systems (such as the kidney and brain) to chemical carcinogens during fetal life vary, extremely, both in quantitative responses to chemical carcinogens and in the nature of tumors induced.

Major Findings:

Cell culture conditions were devised that selectively support growth of 13 or 14 gestation day F344 rat or 11 or 12 gestation day C57BL/6N mouse ureteric bud, precursor of renal collecting duct and inducer of differentiation in metanephrogenic mesenchyme, but inhibit growth of mesenchyme, progenitor of structures proximal to the duct. Isolated buds were cultured in Ham's F12 medium supplemented with epidermal growth factor, selenium, insulin, hydrocortisone, prostaglandin E_1, transferrin, and triiodothyronine; fetal bovine serum (1%) was required for continuous propagation. Cultured rat buds required selenium and epidermal growth factor for survival and growth in culture but also benefitted significantly from medium supplementation with hydrocortisone, and transferrin. Growth of mouse buds, on the other hand, was enhanced with EGF, insulin, and hydrocortisone, but selenium and transferrin had no significant effect. All factors have been maintained in medium preparations for both mouse and rat cells since these factors may influence level of differentiation. Cultured rat bud cells were epithelial in morphology and formed domes. By electron microscopy, many structural characteristics of highly differentiated cells were evident: numerous mitochondria, Golgi apparatus, extensive endoplasmic reticulum, an occasional cilium, intracytoplasmic filaments, polarized formation of microvilli, and gap junctions. Histochemistry revealed considerable functional differentiation as well. Cultured rat bud cells, adult collecting duct and fetal duct anlagen were positive for acid phosphatase, membranelocalized ATPase, and nonspecific esterase. Rat bud cells and fetal duct anlagen expressed high levels of gamma-glutamyl transpeptidase activity while adult collecting duct exhibited slight activity. In addition, immunocytochemical observation of intermediate filament expression revealed the presence of epithelial cytokeratins but absence of mesenchymal vimentin in cultured bud cells and fetal and adult collecting ducts. Cultured mouse bud cells, while epithelial in morphology, appeared as large polygonal cells of mostly cytoplasm or relatively small embryonal cells of primarily nuclear material. Domes have not been observed in confluent cultures. Electron microscopic, histochemical, and immunocytochemical findings were consistent with those of cultured rat bud cells with the exception that mouse fetal duct anlagen and cultured bud cells exhibit no histochemically demonstrable gamma-glutamyl transpeptidase. These results indicate that the culture conditions described can maintain the partially differentiated fetal

collecting duct anlagen in its embryonal state and, therefore, may be useful in culture studies of renal differentiation or studies of heterotypic recombinations with renal embryonal tumor cells. They also identify differences between the rat and mouse in enzyme expression during the developmental period, confirming that these processes are not identical.

Quantitative transplacental dose response curves were determined for a series of chemical carcinogens as previously reported for the Syrian hamster. It was demonstrated that DMBA was the most potent transplacental mutagen at 1500 times control and 1.5-fold greater than the super mutagen NEU. Also NMU is a more potent transplacental mutagen on a molar basis than NEU despite it not being as carcinogenic transplacentally.

Also transplacental mutagenic dose curves were determined for the rat and mouse using NEU as an agent. The transplacental mutagenic effect of x-ray is also being assessed in the Syrian hamster.

The effect of all these agents were determined late in gestation when their carcinogenic effect is strongest. However, the sensitivity of the whole Syrian hamster embryo/fetus to transplacental mutation induction by NEU was previously demonstrated by us to be maximum on day 9 of gestation. The rat with the longer gestation period was determined to have a maximum sensitivity later at day 11.

The phase sensitivity to mutation induction by NEU in different tissues of the Syrian hamster is also being investigated. Preliminary findings indicate the phase sensitivity of cells derived from the central nervous system (under our conditions presumably astrocites) is greatest during the earliest stage of gestation with a second peak at around day 9 to 10. Studies on cells derived from other tissues and grown in selected media are in progress. Another transplacental carcinogen was also determined to be mutagenic to cells of its target organ. DES or diethylstilbestrol has not been found in various in vitro tests to be mutagenic even to Syrian hamster cells to which it was found to be a transforming agent.

In an attempt to investigate the striking differences in carcinogenesis exhibited by various organs among different species, cells are isolated from brain, lung, skin and liver of Syrian hamster, rat and mouse after treatment with DMBA, NEU and NDEA. Using a direct mutagenicity assay substantial mutation induction was found in cells derived from the central nervous system of Syrian hamster treated with all three agents. Studies of other tissues are in progress.

Significance to Biomedical Research and the Program of the Institute:

Studies in rodents have shown that a fetus may be as much as two orders of magnitude more susceptible to carcinogens than an adult of the same species, strain, and sex. The precise reasons for this enhanced vulnerability are not clearly understood, and the fact remains unexplained that, in rodents, carcinogens acting on differentiating fetal tissues principally induce tumors of adult epithelial morphology. Many tumors which develop as a consequence of transplacental exposure to carcinogens are morphologically identical to those inducible in adults. In the mouse kidney, for example, only adenomas and a few carcinomas originating from proximal convoluted tubules develop after prenatal exposure to carcinogens when the kidney is mostly undifferentiated. This suggests that the fundamental genetic damage inflicted on

undifferentiated fetal cells does not preclude subsequent programmed differentiation. The fact that differentiation overrides expression of neoplastic transformation in a given organ system (e.g., the kidney) of certain species such as the mouse, but does not do so in others such as the rat, provides a route to exploration of the basic nature of cellular differentiation to the control of neoplastic growth in the context of prenatal susceptibility to carcinogens.

The ability to determine, experimentally in vitro, the relative potency of transplacental carcinogens to induce mutation in the somatic cells of the fetus is an important methodological advance. Since susceptibility to carcinogens during this time period is greatly increased relative to adults, prenatal testing of putative carcinogens has sometimes been advocated. However, the cost and difficulties of transplacental carcinogenesis experiments would be prohibitive except in some cases. This in vivo/in vitro method would partially fulfill this need.

Second, there is at present no clear explanation of the vast differences in susceptibility to transplacental carcinogens among different organs of different species. Also, vulnerability is specifically time-dependent, being nil in periods before organogenesis and rising to a maximum just before birth. The fundamental question posed by both observations is whether the resulting transplacental tumor incidence is proportional to genetic damage as measured by mutation frequency. One of the alternative explanations is that the genetic damage inflicted by the mutagen initiates, but the controlling factor in tumorigenesis is the process of differentiation and anything that influences that process.

Proposed Course:

Rat renal "blastemal-cell" tumors will continue to be studied in transplantation and in cell and organ cultures to determine whether the morphologically undifferentiated tumor can be induced to form characteristic epithelial structures resembling renal tubules and whether the enzymes characteristic of renal epithelium will develop as markers of morphologically demonstrable differentiation. In organ culture, both natural (ureteric bud) and heterotopic (fetal spinal cord) inductive tissues will be used as potential inducers as well as chemical agents which are known to affect other in vitro systems in which morphogenetic differentiation occurs (cAMP, IUDR, DMSO). Initially, the goal of this program is to determine whether the lesser tendency of fetal rat kidney (in comparison with that of the mouse) to form differentiated epithelial tumors after exposure to transplacental carcinogens is due to interspecies differences in cellular responsiveness to mediators of morphogenetic differentiation.

A major characteristic of the response of rodents to chemical carcinogens during intrauterine development is that susceptibility to neoplastic transformation generally is not demonstrable prior to completion of definitive organogenesis, which marks the beginning of the fetal period of development. True embryos, in which undifferentiated tissues are only beginning to form identifiable organ rudiments, are subject to teratogenic damage but are not, in general, at risk for subsequent tumor development as a consequence of exposure to carcinogens during this stage of development. The question arises whether this indicates that potential neoplastic transformants are generated, but are prevented from expressing their neoplastic genotypes phenotypically by proliferation to generate a tumor. The latter might be accomplished through cell-cell interactions or other mediators of programmed normal differentiation to which cells altered by carcinogens are still responsive.

Mutagenesis in fetal hamster, rat, mouse, and eventually nonhuman primate tissues will be investigated systematically to establish whether genotoxic damage, demonstrable immediately by the mutation assays, correlates with organ-specific and age-dependent transplacental carcinogenesis by various agents in these species. A future project is planned using the fetal hamster in cell cultures from which morphologic transformation of mesenchymal cells is demonstrable. Prenatal hamsters will be subjected transplacentally to a carcinogenic dose of metabolism-independent carcinogen at different stages of development, from implantation of the blastocyst through late fetal life. Cultures prepared from the conceptuses thus exposed will be studied for the presence of transformed cells in an effort to demonstrate the presence of latent transformed cells in fetal tissues that appear refractory to carcinogenesis during early development. The fact that transformation is readily demonstrated in fetal hamster fibroblasts that originate from the soft connective tissues in which tumors are not seen following transplacental exposure to carcinogens strongly suggests that such an approach will be fruitful.

Publications:

Perantoni, A., Kan, F., Dove, L. F. and Reed, C. D.: Selective growth in culture of fetal rat renal collecting duct anlagen: Morphologic and biochemical characterization. Lab. Invest. (In Press)

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05299-04 LCC

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Interspecies Differences in Transplacental Carcinogenesis and Tumor Promotion

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

	Name	Title	Lab	Institute
PI:	B. A. Diwan	Expert	LCC	NCI
Others:	J. M. Rice	Chief	LCC	NCI
	J. M. Ward	Chief, Tumor Pathology and Pathogenesis Sec.	LCC	NCI
	L. M. Anderson	Expert	LCC	NCI
	L. K. Keefer	Chief, Chemistry Section	LCC	NCI
	P. Blumberg	Chief, Molec. Mech. of Tumor Promotion Section	LCCTP	NCI
	A. Hagiwara	Guest Researcher	LCC	NCI

COOPERATING UNITS (if any)

Microbiological Associates, Inc., Bethesda, MD (M. L. Wenk)

LAB/BRANCH

Laboratory of Comparative Carcinogenesis

SECTION

Tumor Pathology and Pathogenesis Section

INSTITUTE AND LOCATION

NCI, NIH, Frederick, Maryland 21701

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.2	1.6	0.6

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Tumor promotion phenomena in two-stage carcinogenesis were systematically explored in various rodent species in conjunction with transplacental carcinogenesis. The relationship between molecular structure and promoting activity of various barbiturates, phthalic acid esters and benzodiazepine tranquilizers is investigated by sequential administration to animals of a transient, low level exposure to a genotoxic carcinogen followed by the test agent under study. Organ specificities and interstrain and interspecies correlations in tumor promotion are investigated for clues to the mechanism(s) of action of tumor promoters. Barbital has been found to promote carcinogenesis in the rat renal cortical epithelium, as well as in the liver while phenobarbital was found to promote carcinogenesis in the liver and thyroid follicular parenchyma. Phenobarbital also promoted hepatocarcinogenesis in adult mice but inhibited the same when the offspring were exposed to this drug before they became sexually mature. Long acting barbiturates, phenobarbital and barbital, promoted carcinogenesis in different tissues in mice and rats exposed transplacentally to N-alkylnitrosoureas and revealed a greater extent of initiation at several "minor" sites than expected from tumor incidence data from offspring not given the promoters. A sequence of multiple low doses of nitrosomethylurea was found to be a useful broad spectrum tumor initiation regimen. The plasticizer, di-2-ethylhexylphthalate was found to promote transformation of JB6 mouse epidermal cells and act as a second stage promoter of mouse skin. Benzodiazepine tranquilizers, diazepam and oxazepam are strong promoters of hepatocarcinogenesis in adult mice. Premalignant JB6 mouse epidermal cells are used to investigate the mechanisms of tumor promotion by various drugs and environmental pollutants.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

B. A. Diwan	Expert	LCC	NCI
J. M. Rice	Chief	LCC	NCI
J. M. Ward	Chief, Tumor Pathology and Pathogenesis Section	LCC	NCI
L. M. Anderson	Expert	LCC	NCI
L. K. Keefer	Chief, Chemistry Section	LCC	NCI
P. Blumberg	Chief, Molec. Mech. of Tumor Promotion	LCCTP	NCI
A. Hagiwara	Guest Researcher	LCC	NCI

Objectives:

1) To investigate the relationship between molecular structure and promoting activity of various barbiturates and benzodiazepine tranquilizers available commercially for human therapeutics, 2) to determine the cell- and organ-specific tumor promoting effects of barbiturates and the plasticizer, di-2-ethylhexylphthalate and its metabolites in various rodent species and to explore the mechanisms underlying such activities, 3) to characterize and systematically define the limits of organ, strain and species specificities of promotion by these compounds, 4) to demonstrate the occurrence of potentially neoplastic (initiated cells) in organs and tissues which appear refractory to tumor development by transient transplacental or systemic exposure to directly acting or pulse carcinogens alone, and 5) to utilize promotable JB6 mouse epidermal cells to investigate the mechanisms of the promoting action of these agents.

Methods Employed:

In transplacental carcinogenesis studies, precisely timed, pregnant mice, rats and Syrian hamsters are exposed to chemical carcinogens at defined periods during gestation. Offspring from these mothers and young and adult animals exposed to carcinogens by conventional routes are subsequently exposed to nongenotoxic agents known or suspected to promote tumorigenesis in one or more organs. Preneoplastic proliferative lesions and neoplasms resulting from such treatments are identified and classified by histological, histochemical and ultrastructural parameters. For routine quantitative evaluations of preneoplastic focal proliferative lesions in the liver, an automated image analyzer (Videoplan, Carl Zeiss, Inc., NY) and Zeiss Stereology Software are used. Barbiturates and hydantoins of desired molecular structure are synthesized and purified in the Chemistry Section of LCC. For determination of biochemical effects of both initiators and promoters on various animal tissues, high performance liquid chromatography (HPLC), spectrophotofluorometric methods, and different radioimmunoassays are routinely used. Premalignant JB6 mouse epidermal cells are exposed to suspected tumor promoters and the progression of these cells to tumor cell phenotypes is measured by colony formation in soft agar at 14 days. Ultrastructural changes accompanying promoter-mediated phenotypic transformation are characterized by scanning and transmission electron microscopy.

Major Findings

A comparison of hepatic tumor yields in male F344 rats given N-nitrosodiethylamine (DEN) followed by different barbiturates showed that both phenobarbital and barbital are strong liver tumor promoters while amobarbital, an intermediate acting compound, and the pharmacologically inactive barbituric acid are totally ineffective as enhancers of hepatocarcinogenesis, failing to increase the final tumor incidence or the multiplicity of tumors. In addition to the enhancement of liver tumorigenesis, barbital promoted the development of renal epithelial tumors while phenobarbital promoted the development of thyroid follicular cell neoplasms. Both phenobarbital and barbital increased liver weights, and indeed hepatic cytochrome P-450 and cytochrome P-450-dependent monooxygenases. Little or no effect on either liver weights or hepatic cytochrome P-450 levels was observed with amobarbital or barbituric acid.

Experiments designed to test the promoting effects of phenobarbital, barbital, amobarbital and barbituric acid on intestinal carcinogenesis initiated by methylacetoxymethylnitrosamine (DMN-OAC) in male F344 rats clearly demonstrated that none of these barbiturates promote intestinal tumors initiated by this agent. Phenobarbital, however, enhanced the incidence of thyroid follicular epithelial tumors in DMN-OAC-treated rats. Barbital, on the other hand, significantly enhanced renal carcinogenesis in rats following DMN-OAC treatment. Histologically, the renal tumors were both renal cortical tubular adenomas and pelvic transitional cell papillomas and carcinomas.

Effects of concurrent and/or subsequent administration of phenobarbital on carcinogenesis initiated by a direct acting alkylating agent, nitrosomethylurea (NMU), were also investigated in F344 rats. The administration of phenobarbital either simultaneous with or subsequent to NMU treatments enhanced thyroid tumorigenesis in rats. Phenobarbital given subsequent to NMU significantly enhanced liver tumorigenesis in rats of both sexes given NMU in four divided doses. No liver tumors occurred in males or females given NMU alone. Phenobarbital did not affect the incidence of any other kind of neoplasms seen in NMU-initiated or control rats.

Studies were designed to investigate the tumor-promoting effects of phenobarbital and deoxycholic acid in male Syrian Golden hamsters following initiation with either DEN or methylazoxymethanol acetate (MAM). Our preliminary results suggest that MAM is a more effective initiator of hepatocarcinogenesis than DEN and both phenobarbital and deoxycholic acid are ineffective as promoters of hepatocarcinogenesis in hamsters.

Adult inbred strains of mice were found to differ significantly in their susceptibility to two-stage liver carcinogenesis initiated by DEN and promoted by phenobarbital. Mice of strains DBA/2N and C3H/HeN were susceptible and those of strain C57BL/6N were relatively resistant, while NFS/N mice were totally refractory to two-stage liver carcinogenesis. C57BL/6N mice were susceptible to DEN initiation but resistant to liver tumor promotion by phenobarbital. Studies are in progress to investigate the genetic as well as biochemical mechanisms responsible for these strain differences to two-stage liver carcinogenesis in mice.

The plasticizer, di-2-ethylhexylphthalate (DEHP), a confirmed promoter of hepatocarcinogenesis, was found to promote transformation in JB6 mouse epidermal cells and act as a second stage promoter on mouse skin. Unlike some well-known skin tumor

promoters, however, DEHP does not compete for TPA receptors and may act through different mechanisms. The possible mechanism(s) of DEHP promotion is being explored. Of the two major metabolites of DEHP tested, mono-2-ethylhexylphthalate promoted transformation of JB6 cells but 2-ethylhexanol was ineffective.

Effects of postnatal administration of phenobarbital on the development of tumors initiated by prenatal NMU were investigated in rats. The results of this experiment showed that NMU, in addition to being a complete transplacental carcinogen for rat kidney and nervous system, is a strong initiator of thyroid tumorigenesis in male offspring and also a relatively weak initiator of preneoplastic lesions in female offspring. Thyroid tumors and liver lesions were observed only in offspring that received NMU prenatally and phenobarbital postnatally.

Transplacental exposure to N-nitrosoethylurea (ENU) causes chiefly intestinal carcinomas and lung tumors in B10.A mice and presumably initiates carcinogenesis in other tissues. To investigate organ-specific tumor promotion by barbiturates, pregnant B10.A mice were given ENU on day 18 of gestation and offspring received phenobarbital, barbital, amobarbital or barbituric acid in drinking water during postnatal weeks 5 through 30. None of the barbiturates significantly affected the incidence or multiplicity of intestinal or pulmonary tumors. Barbital significantly increased the incidence of renal cortical adenomas and carcinomas while phenobarbital tripled the incidence of thyroid follicular cell neoplasms in ENU-treated offspring. Phenobarbital, however, inhibited hepatocellular carcinogenesis in male offspring. Amobarbital or barbituric acid had no effects on tumor incidence in any organ. Thus, transplacental administration of chemical carcinogens followed by postnatal application of tumor promoters can result in tumor formation at sites where no tumor would occur in absence of promotion.

Studies on tumor promoting effects of benzodiazepine tranquilizers, diazepam and oxazepam are in progress. However, the results of the experiment in which the male mice were sacrificed at 28 weeks of age (i.e., 21 weeks on promotion diet) clearly showed that both diazepam and oxazepam promote the development of hepatocellular neoplasms. Diazepam is more effective than oxazepam and the effects are proportional to dose.

Significance to Biomedical Research and the Program of the Institute:

Barbiturates are widely used as hypnotic, anticonvulsant and sedative drugs while benzodiazepine (diazepam, oxazepam) tranquilizers are routinely used in the treatment of anxiety, tension and other minor emotional disorders. Rigorous analyses of the relationship between the structure and promoting activity of various barbiturates and hydantoins and the determination of the organ and species specificity of these agents will provide clues as to their mechanisms of action as tumor promoters. If organ-specificity and dosage requirement of these drugs prove to be consistent in different animal species including nonhuman primates, it may be possible to predict the effects of such agents in man. In view of the importance and widespread use of a plasticizer, di-2-ethylhexylphthalate, it is important to determine its mechanism(s) of action and examine its effects on tumor development in various tissues of different mammalian species. The demonstration of tumor-promoting activity of environmental pollutants and therapeutic drugs at therapeutic dosage levels is of obvious significance to public health if such promoting action is not limited to rodent species only.

Proposed Course:

Systemic analyses of the relationship between structure and promoting activity of different barbiturates will be extended to gain further insight into the relationship between their structure and promoting activity. Barbiturates with appropriate molecular structures have been synthesized in the Chemistry Section of our laboratory and are being tested for their tumor promoting activities. These experiments are designed to answer two specific questions: 1) Is long acting/anticonvulsant activity characteristic of promoting compounds? 2) Do anticonvulsive barbiturates lacking hypnotic activity promote carcinogenesis? Hydantoins (phenantoin and its N-dimethylated metabolite, Nirvanol) are nonhypnotic anticonvulsants. Hydantoin derivatives are also being studied for their tumor promoting effects in rats.

Tumor promoting and organ-specificity of each barbiturate will be investigated in at least three rodent species--mouse, rat and hamster. If organ-specificity of barbiturates varies with the structure and if it is consistent in all rodent species, these studies will be extended to nonhuman primates. Biochemical mechanisms for genetic (strain) differences in tumor promotion will be determined. In order to analyze the genetic mechanism(s) responsible for strain differences in response to phenobarbital promotion, a Mendelian hybridization experiment is being performed with mice of susceptible (DBA/2) and resistant (C57BL/6) strains, the first and second filial generation hybrids (F1, F2) and backcross to the two parental strains. Studies are also in progress to investigate the tumor promoting effects of barbital (which is not metabolized significantly by liver or any other organ) in liver of various strains of mice used in our earlier studies. Studies are in progress to determine the short- and long-term effects of diazepam and oxazepam on various morphological and biochemical parameters in mouse liver. Studies employing promotable JB6 cell models will be extended to the investigations of the mechanisms of tumor promotion by suspect drugs and environmental pollutants. These studies will be carried out in consultation and collaboration with other sections in the LCC: Office of the Chief, Chemistry Section, Perinatal Carcinogenesis Section and Ultrastructural Studies Section.

Publications:

Diwan, B.A., Rice, J.M., Ohshima, M., Ward, J.M. and Dove, L.F.: Comparative tumor-promoting activities of phenobarbital, amobarbital, barbital sodium, and barbituric acid on livers and other organs of male F344/NCr rats following initiation with N-nitrosodiethylamine. JNCI 74: 509-516, 1985.

Diwan, B.A., Ward, J.M., Henneman, J. and Wenk, M.L.: Effects of short-term exposure to the tumor promoter, 12-O-tetradecanoylphorbol-13-acetate on skin carcinogenesis in SENCAR mice. Cancer Lett. 25: 177-184, 1985.

Diwan, B.A., Ward, J.M., Rice, J.M., Colburn, N.H. and Spangler, E.F.: Tumor-promoting effects of di(2-ethylhexyl)phthalate in JB6 mouse epidermal and mouse skin. Carcinogenesis 6: 343-347, 1985.

Ward, J.M., Diwan, B.A., Ohshima, M., Hu, H., Schuller, H.M. and Rice, J.M.: Tumor initiating and promoting activities of di(2-ethylhexyl)phthalate *in vivo* and *in vitro*. Environ. Hlth. Perspect. (In Press)

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05301-04 LCC

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Biology and Pathology of Natural and Experimentally Induced Tumors

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	J. M. Ward	Chief, Tumor Pathology and Pathogenesis Section	LCC	NCI
Others:	L. Anderson	Expert	LCC	NCI
	C. Reynolds	Senior Staff Fellow	BTB	NCI
	A. Palmer	Research Veterinarian	LCC	NCI
	A. Hagiwara	Guest Researcher	LCC	NCI
	U. Rapp	Chief, Viral Pathology Section	LVC	NCI
	S. Rehm	Visiting Associate	LCC	NCI
	J. Junker	Staff Fellow	LCC	NCI

COOPERATING UNITS (if any)

Veterans Administration Hosp., Pittsburgh, PA (G. Singh); University of Texas Health Science Center, Houston, TX (R. Pardue)

LAB/BRANCH

Laboratory of Comparative Carcinogenesis

SECTION

Tumor Pathology and Pathogenesis Section

INSTITUTE AND LOCATION

NCI, NIH, Frederick, Maryland 21701

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.5	2.5	

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The pathology and biology of experimentally induced and naturally occurring neoplasms of rodents are characterized and compared using serial sacrifice studies, avidin-biotin (ABC) immunocytochemistry, automated image analysis with stereology, conventional light microscopy, ultrastructure and histochemistry. Histopathogenesis investigations were performed for mouse and hamster liver, mouse and rat lung, and sarcomas induced by Harvey sarcoma virus. Ras-Ha p21 was found by ABC immunocytochemistry to be present on the cell membranes of sarcoma cells, erythroblasts and reticulum cells in viral infected mice but not in other normal cells using a sheep IgG to a peptide in p21. Using this antiserum we could not demonstrate immunoreactive p21 in any other tumors, spontaneous or induced, even some chemically-induced tumors with demonstrably transfectable Ha-Ras. The vast majority of early or late pulmonary tumors in rats and mice were immunoreactive for the apoproteins of pulmonary surfactant but never Clara cell antigen. This finding suggested that the alveolar Type II cell is the origin of lung tumors of rats and mice. Some lung tumors of mice induced by ethylnitrosourea demonstrated no reactive antigens. The phenotype of mouse liver tumors initiated and/or promoted by chemicals was found to depend on the promoters. For example, di(2-ethylhexyl)phthalate promoted basophilic tumors while phenobarbital and diazepam promoted eosinophilic tumors.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

J. M. Ward	Chief, Tumor Pathology and Pathogenesis Section	LCC	NCI
L. M. Anderson	Expert	LCC	NCI
C. Reynolds	Senior Staff Fellow	BTB	NCI
A. Palmer	Research Veterinarian	LCC	NCI
A. Hagiwara	Guest Researcher	LCC	NCI
U. Rapp	Chief, Viral Pathology Section	LVC	NCI
S. Rehm	Visiting Associate	LCC	NCI
J. Junker	Staff Fellow	LCC	NCI

Objectives:

To characterize the biology and pathology of naturally occurring and experimentally-induced tumors of laboratory animals.

To identify differences and significance of differences between naturally occurring and induced tumors.

Methods Employed:

The avidin-biotin peroxidase-complex (ABC) technique has been utilized to localize cell specific antigens and to follow tumor development by antigen localization. We have shown that tumor and normal cells may contain antigens for several reasons: antigen production and storage, leakage of serum proteins, infiltration of nonneoplastic cells into tumors and tissues, phagocytosis or membrane coating of antigen in an aqueous environment. Antigens may also be characterized by their specificity and significance in cells: cell specific antigens such as hormones and surface antigens; new antigens such as AFP, transforming gene proteins, carcino-embryonic antigen; antigen loss such as differentiation antigens (enzymes, proteins, cell surface glycoproteins) and abnormal antigen distribution, especially for intracellular filaments. Our most significant findings include antigen expression and origin of pulmonary bronchiolar/alveolar tumors in rats and mice, expression of transforming gene protein products in induced and natural tumors, and normal tissues and differentiation expression of alphafetoprotein in preneoplastic, benign and malignant hepatocellular lesions of rats, mice and monkeys.

We have used several monoclonal and polyclonal antibodies to transforming gene protein products or to their peptides (synthetic), which originated from NCI investigators or commercial sources, to study the expression of the antigens in various stages of tumor progression in an effort to evaluate their significance in tumor progression.

Major Findings:

Many of the antibodies to oncogene protein products were not immunoreactive in fixed tissue sections with tumors which contain enhanced expression of oncogenes detected by transfection or immunoblotting. We have found that two polyclonal antibodies to synthetic peptides provided us with our most consistent findings. One antibody to a

peptide containing 20 amino acids in the carboxy terminal portion of mouse Ras^{Ha} p21 has provided us with evidence that p21 expression is limited primarily to neoplastic mesenchymal cells (fibroblasts, endothelium) in mice injected with Harvey sarcoma virus/Moloney leukemia virus, although we have found that some nonneoplastic cells express the cell surface antigen as well, including splenic erythroblasts and reticular cells in lymph nodes. With another polyclonal antibody to a peptide in v-Raf, we have shown that antigen expression can be found on the cell surface of benign liver tumor cells in mice. In collaborative studies with Drs. S. Aaronson and A. Eva of NCI, we have shown that Ha-Ras oncogene activation can be demonstrated in experimentally-induced benign and malignant mouse liver tumors.

Alphafetoprotein (AFP) has been used as an oncofetal protein marker for hepatocellular carcinoma in various species. In our various studies of tumor promotion in rats, mice and monkeys, we have demonstrated by ABC immunocytochemistry that AFP expression in the various stages of carcinogenesis is species related. In mice, AFP can be found on hepatocytes in small preneoplastic lesions, adenomas and carcinomas. There seems to be, however, increased expression in the malignant tumors. In rats and humans AFP has only been found in carcinomas. In Patas monkeys, we recently found that AFP is expressed in both benign and malignant tumors, but not in preneoplastic lesions.

We have shown for the first time that the apoproteins of pulmonary surfactant apoprotein (SAP) can be used as a marker of alveolar Type II tumor histogenesis in rats and mice. In mice with spontaneous or induced pulmonary lesions, the majority of alveolar cells in small focal alveolar hyperplasias contained SAP while larger nodular adenomas usually contained the antigen, but the largest pulmonary tumors had focal loss of SAP. Thus, alveolar Type II cells were probably the origin of the majority of natural and induced tumors of mice although some lesions arose within or adjacent to bronchioles. Some investigators have provided evidence that ethylnitrosourea induced Clara cell tumors when given transplacentally. We have shown that these tumors do not contain Clara cell antigen while they contain SAP. In addition, in our most recent studies, we have shown that the majority of these tumors arise within alveolar walls and possibly from Type II cells. These studies have been supported by ultrastructural investigations of selected individual lesions. Similar findings were seen for NMU-induced lung tumors in F344 rats.

Significance to Biomedical Resesarch and the Program of the Institute:

Rodent tumors are used by investigators for assessment of the carcinogenic effects of chemicals, studies on the mechanisms of carcinogenicity or the role of modifiers of carcinogenesis, and in safety tests for chemicals required by U.S. regulatory agencies. Knowledge of the nature, morphology and natural history of both natural and induced tumors is necessary for careful and accurate evaluations of, and regulatory decisions based on, these animal experiments and their uses. Expression of oncogene protein products in various stages of tumor progression should allow us to better understand the role, nature and function of these proteins.

Proposed Course:

(1) We will pursue the immunocytochemical localization of transforming gene protein products in the various stages of tumor progression using the antibodies and model

systems which provide the most useful information. We will also attempt to determine the reasons why antibodies specific to synthetic peptides or proteins are not immunoreactive with antigens we know are present in fixed tissue sections. Preliminary evidence has shown that the manner of fixation and the fixative itself are important. We will extend our observations and provide quantitative evidence of our findings. We will also use ultrastructural immunocytochemistry to localize the antigens on the cell surface and in cell organelles in collaboration with Dr. James Junker of the USS, LCC.

(2) We will concentrate on tumor histogenesis studies in mouse lung using image analysis and immunocytochemistry in the transplacental ENU model system.

Publications:

Goodman, D. G., Ward, J. M. and Reichardt, W. D.: Splenic fibrosis and sarcoma in F344 rats fed diets containing aniline, P-choloroaniline, azobenzene, o-toluidine, 4,4'-sulfonyldianiline or D&C red 9. JNCI 73: 265-273, 1984.

Hall, W. C. and Ward, J. M.: A comparison of the avidin-biotin-peroxidase complex (ABC) and peroxidase-anti-peroxidase (PAP) immunocytochemical techniques for demonstrating Sendai virus infection in fixed tissue specimens. Lab. Anim. Sci. 34: 261-263, 1984.

Ohshima, M., Ward, J. M., Singh, G. and Katyal, S. L.: Immunocytochemical and morphological evidence for the origin of N-nitrosomethylurea-induced and naturally-occurring primary lung tumors in F344/NCr rats. Cancer Res. (In Press)

Ohshima, M., Ward, J. M. and Wenk, M. L.: Preventive and enhancing effects of retinoids on the development of naturally occurring tumors of skin, prostate gland and endocrine pancreas in aged male ACI/SegHapBr rats. JNCI 74: 517-524, 1985.

Reynolds, C. W. and Ward, J. M.: LGL lymphoproliferative diseases in man and experimental animals. A summary of these cells; their characteristics and potential experimental uses. In Lotzova, E. and Herberman, R. B. (Eds.): Immunobiology of Natural Killer Cells. West Palm Beach, CRC Press. (In Press)

Singh, G., Katyal, S. L., Ward, J. M., Gottron, S. A., Wong-Chong, M. and Riley, E. J.: Secretory proteins of the lung in rodents: immunocytochemistry. J. Histochem. Cytochem. 33: 564-568, 1985.

Ward, J. M.: Morphology of potential preneoplastic hepatocyte lesions and liver tumors and a comparison with other species. In Popp, J. A. (Ed.): Current Perspectives in Mouse Liver Neoplasia. Washington, D.C., Hemisphere Press, pp. 1-26, 1984.

Ward, J. M.: Proliferative lesions of the glandular stomach and liver in F344 rats fed diets containing Aroclor 1254. Environ. Health Perspect. (In Press)

Ward, J. M., Singh, G., Katyal, S. L., Anderson, L. M. and Kovatch, R. M.: Immunocytochemical localization of the surfactant apoprotein and Clara cell antigen in chemically induced and naturally occurring pulmonary neoplasms of mice. Amer. J. Pathol. 118: 493-499, 1985.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05303-04 LCC

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Pathogenesis and Promotion of Natural and Induced Tumors

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	J. M. Ward	Chief, Tumor Pathology and Pathogenesis Section	LCC	NCI
Others:	B. A. Diwan	Expert	LCC	NCI
	S. Rehm	Visiting Associate	LCC	NCI
	A. Hagiwara	Guest Researcher	LCC	NCI
	H. S. Hu	Staff Fellow	LCC	NCI
	L. K. Keefer	Chief, Chemistry Section	LCC	NCI
	H. Schuller	Acting Chief	LETM	NCI

COOPERATING UNITS (if any)

Microbiological Associates, Inc., Bethesda, MD (Drs. M. Wenk and F. Spangler)

LAB/BRANCH

Laboratory of Comparative Carcinogenesis

SECTION

Tumor Pathology and Pathogenesis Section

INSTITUTE AND LOCATION

NCI, NIH, Frederick, Maryland 21701

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.0	2.0	

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Di(2-ethylhexyl)phthalate (DEHP) and phenobarbital (PB) were studied in model systems for initiation and promotion in skin, mouse and rat liver, and JB6 mouse epidermal cells. DEHP was found to be a liver tumor promoter, a second stage skin tumor promoter, a "weak" complete skin tumor promoter and a promoter of JB6 cells to anchorage independence in mice. It promoted skin tumors even only after thirty days of dietary exposure for the mouse. PB was also a tumor promoter in mouse liver. DEHP, however, was not a tumor promoter in rat liver despite its similar hepatotoxicity in rats and mice.

PHS 6040 (Rev. 1/84)

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

J. M. Ward	Chief, Tumor Pathology and Pathogenesis Section	LCC	NCI
B. A. Diwan	Expert	LCC	NCI
S. Rehm	Visiting Associate	LCC	NCI
A. Hagiwara	Guest Researcher	LCC	NCI
H. S. Hu	Staff Fellow	LCC	NCI
L. K. Keefer	Chief, Chemistry Section	LCC	NCI
H. Schuller	Acting Chief	LETM	NCI

Objectives:

To characterize the sequential morphologic and biologic steps in the development of cancer after initiation and/or promotion and to systemically study the nature of cellular and organ specificities and species differences in response to tumor promoters.

Methods Employed:

Di(2-ethylhexyl)phthalate, a plasticizer and hepatic peroxisome proliferator, is a hepatocarcinogen in rats and mice as demonstrated in routine lifetime studies. Because DEHP was demonstrated to have no genotoxic activity in bacterial mutagenesis assays or in other in vitro assays, the hypothesis was tenable that this compound achieved its biologic effects by acting as a tumor promoter, enhancing the development of naturally occurring liver tumors.

At least in the mouse skin system, the promotion stage has empirically been subdivided into two distinct components, Stage I and Stage II, which are qualitatively different from initiation and from each other. The structural analog of phorbol esters, mezerein, is only a weak complete promoter, but when given repeatedly (2x/wk) after limited exposure to 12-O-tetradecanoylphorbol-13-acetate (TPA), it induces a significant tumor response in a dose-dependent manner. No liver carcinogens or promoters were demonstrated to be active in this system. There are few in vitro assays for tumor promoters, and only one that is predictive of target cell specificity in vivo. The JB6 system was originally developed to study promotion by phorbol esters, and is based on induction by certain classes of chemicals (e.g., complete and second stage promoters) of transformation of mouse epidermis-derived cell lines to a neoplastic phenotype, characterized by anchorage independence and tumorigenicity. To investigate further the mechanism of DEHP carcinogenesis, we investigated the tumor-promoting effect of this plasticizer in rat and mouse liver and mouse skin systems in vivo and in an in vitro assay using promotable JB6 mouse epidermal cell lines.

Study of initiating and promoting activity of DEHP in rat and mouse liver. We developed a simple system to study the initiating or promoting activity of chemicals in mouse or rat liver. Weanling male B6C3F1 mice or female F344 rats were injected once i.p. with N-nitrosodiethylamine (80 or 200 mg/kg at 4-5 weeks of age and placed

on diets or water containing the promoter (DEHP, 3000, 6000 or 12000 ppm; or PB, 500 ppm) 1-2 weeks later. The promoter exposure time varied from 1 day to 18 months. For determination of initiating activity, mice were given one intragastric dose of DEHP (25 or 50 gm/kg) and placed on phenobarbital 1-2 weeks later. Animals were sacrificed at varying intervals from 2-18 months later. The number and size of hepatocellular focal proliferative lesions including adenomas and carcinomas was quantitated using an automated image analyzer (Videoplan, Zeiss, Inc., NY, NY).

Skin initiation-promotion studies. CD-1 mice initiated by a single topical application of 50 µg DMBA to the dorsal skin received DEHP (98.1 mg in acetone, 0.2 ml total volume) or TPA (10 µg in 0.2 ml acetone) twice weekly for 40 weeks and were sacrificed at 40 weeks in a routine skin initiation-promotion protocol. To test for second stage promoting activity of DEHP, female SENCAR mice were given DMBA once (20 µg), and then TPA (2 µg, twice a week for 2 weeks), followed by DEHP (100 mg, twice weekly) or by TPA, mezerein or acetone weekly for up to 26 weeks. To test for complete promoting activity by DEHP in SENCAR mice, DEHP was given twice weekly after a single dose of DMBA (20 µg). Mice were sacrificed at 28 weeks.

Anchorage independence induced in JB6 mouse epidermal cells. JB6 cell lines C141, C121 and R219 were used to investigate the promoting ability of DEHP; the C141 cell line was utilized to determine the promoting ability of mono(2-ethylhexyl)phthalate (MEHP) and 2-ethylhexanol (EH). Cell cultures were grown in monolayer culture in EMEM containing 80% fetal calf serum (FCS) and antibiotics. JB6 cells were suspended in culture medium containing 0.33% Difco agar at a temperature less than 40°C to which solvent alone or stock solutions of DEHP, MEHP or EH had been added. The suspension of 1-5 ml, containing 10^4 cells and 1.5 µl of test solution per 60 mm petri dish, was layered over 0.5% agar base. Assays were carried out in duplicate and in two different laboratories (LCC by Dr. Diwan and LVC by Dr. Colburn) at 10% FCS concentrations. Colonies were counted at 14 days as described previously.

Major Findings:

Promotion of liver tumors. DEHP significantly promoted focal hepatocellular proliferative lesions (FHPL), including adenomas and carcinomas in mice, but not in rats. Our negative findings in rats are in agreement with the recent reports of other investigators studying DEHP and other peroxisomal proliferators. We also demonstrated the timed sequence of tumor initiating and promoting effects of DEN, DEHP and PB. We could readily show that the time of sacrifice was important for concluding whether the initiator or promoter induced FHPL. Moreover, DEHP was shown to be an effective promoter after only 30 days exposure while PB was not. FHPL promoted by DEHP were larger than those promoted by PB suggesting a mitogenic effect on the cells in the lesions. PB-promoted foci were histologically eosinophilic suggesting smooth endoplasmic reticulum proliferation and a direct effect of PB on tumor cells or phenotypic evidence of a genetic change. DEHP-promoted lesions did not have marked peroxisomal hyperplasia as did nonneoplastic hepatocytes. PB promoted liver foci and tumors in mice only when the initiator, DEN, was injected at 30 days of age, but not at 15 days of age, when liver cell proliferation is marked.
Skin tumor promotion. DEHP failed to initiate or promote skin carcinogenesis in CD-1 mice even though the concentration of DEHP used was several orders of magnitude higher than those required for DMBA as an initiator and TPA as a promoter. In female SENCAR mice, DEHP was a "weak" complete promoter but had significant second-stage promoting activity on skin carcinogenesis and less activity than

mezerein. As a consequence of using controls exposed to TPA for 2 weeks (4 applications), we found that TPA can promote skin tumors initiated by DMBA after only 1-4 applications of TPA. Tumors promoted by these few applications of TPA do not regress in contrast to those occurring after repeated exposure to TPA, many of which regress.

Epidermal cell culture. DEHP showed activity for promotion of transformation in three promotable (P+) JB6 clonal lines of mouse epidermis-derived cells. These lines of JB6 cells including C141, C121 and R219 have previously been shown to be promoted to anchorage independence and tumorigenicity by tumor promoting phorbol esters, and also by mezerein, benzoyl peroxide and epidermal growth factor. Of the three cell lines used, C141 showed the most pronounced maximum response to DEHP; nearly 32% of cells gave rise to colonies in 10% serum medium in the presence of DEHP at final concentration of 26×10^{-6} M. MEHP, a major hydrolysis product of DEHP, was much more toxic than the parent compound and concentrations above 6×10^{-8} M were toxic to JB6 cells. MEHP concentrations shown to be effective ranged from 2 to 5×10^{-8} M. A second hydrolysis product of DEHP, EH, however, failed to promote transformation.

Significance to Biomedical Research and the Program of the Institute:

The commonly used plasticizer, DEHP, was (a) a strong promoter of hepatocellular tumors initiated by DEN in mice; (b) a second stage skin tumor promoter and a weak complete promoter in SENCAR mouse skin after DMBA initiation, and (c) also an inducer of anchorage independence in promotable mouse JB6 epidermal cells. We and others have shown in our earlier studies that JB6 cells do not respond to promoters of carcinogenesis that are effective in tissues other than mouse skin, such as barbiturates or methapyrilene. DEHP is the only promoter of hepatocarcinogenesis known to be active in this system. It has been generally believed that compounds that are promoters in one system may be inactive in another. For example, deoxycholic acid, a promoter of colon tumor in rats and PB, a promoter of liver tumor in rats and mice, are inactive as promoters in mouse skin. On the other hand, TPA has been shown to promote squamous cell tumors morphologically similar to those of skin in both forestomach and vagina in mice. Recent studies with PB also show that in addition to being a promoter of hepatocarcinogenesis, this drug also enhances thyroid follicular cell neoplasms. Our results on the promoting ability of DEHP in at least two distinct kinds of epithelium (e.g., liver and skin) are also at variance with the postulated organ specificity of promoters.

The tumor promoting effects of DEHP in mice suggest an effect on initiated cells during the first 30 days after DEN exposure and also a mitogenic effect on proliferative lesions after they were seen histologically later in the experiment. Our findings suggest that DEHP may act at two stages in the carcinogenic process, one involving gene expression early in the process of multistage carcinogenesis, and the second after the appearance of the histological proliferative lesions and involving a mitogenic affect of DEHP on preneoplastic and tumor cells. The mitogenicity of phthalate esters is well known at least in normal hepatocytes. The role of DEHP on gene expression is not known. In collaboration with Drs. A. Eva and S. Aaronson (NCI, LCMB), we have found that a large proportion (30-50%) of DEN-induced hepatocellular adenomas and carcinomas have an activated Ha-Ras oncogene. Thus, it is conceivable that DEHP acts, in some way, on Ras^{Ha} oncogene expression. We have also shown by immunocytochemistry that v-Raf transforming protein is expressed by benign liver tumor cells but not in normal cells. While DEHP is a mitogen for rat

liver cells and causes peroxisomal proliferation, it is not a liver tumor promoter in rats. Thus, factors other than hyperplasia and peroxisomal proliferation appear to be involved in liver tumor promotion in rats. A major metabolite of DEHP in mice and humans, MEHP, occurs only in trace amounts in rats. Thus, MEHP may play a role in carcinogenesis and tumor promotion in mice.

Proposed Course:

The possible mechanism(s) of tumor promotion by DEHP will be investigated using our in vivo mouse and rat liver model and in vitro JB6 epidermal cells. MEHP will be synthesized at FCRF to use in similar studies. It has been suggested that peroxisomal proliferators as a group may be carcinogenic by a nongenotoxic mechanism. There is some evidence, however, that suggests that free oxygen radicals and lipid peroxidation play roles in carcinogenesis by these compounds and others. To investigate whether this mechanism applies to DEHP carcinogenesis also, antioxidants ethoxyquin and 2(3)-tert-butyl-4-hydroxyanisole (BHA) will be administered simultaneously with DEHP to mice initiated with DEN. If the mechanism of action of DEHP is similar to other peroxisomal proliferators, the antioxidants would effectively inhibit tumor promotion caused by DEHP. We plan to study their effect on peroxisomal proliferation and DNA damage induced by proliferated peroxisomes in rats and mice.

Generation of reactive oxygen may play a role in DEHP tumor promotion. We plan to examine the effects of enzyme modifiers (BHT, BHA) or inhibitors of free radical generation and antioxidants (BHA, BHT, Uric acid) on induction of anchorage independent transformation by DEHP in JB6 epidermal cells to evaluate the role of species of reactive oxygen such as O_2, H_2O_2, OH, $^1O_2$ and lipid peroxides in the promotion process. The effect of DEHP on gene expression, especially Ras^{Ha} oncogene, will be studied through collaborative efforts with Drs. Eva and Aaronson. Also, the role of a major metabolite of DEHP, MEHP, in tumor promotion and oncogene expression will be studied.

Publications:

Diwan, B. A., Ward, J. M., Henneman, J. and Wenk, M. L.: Effects of short-term exposure to the tumor promoter, 12-O-tetradecanoylphorbol-13-acetate on skin carcinogenesis in SENCAR mice. Cancer Lett. 26: 177-184, 1985.

Diwan, B. A., Ward, J. M., Rice, J. M., Colburn, N. H. and Spangler, E. F.: Tumor-promoting effects of di(2-ethylhexyl)phthalate in JB6 mouse epithelial cells and mouse skin. Carcinogenesis 6: 343-347, 1985.

Schuller, H. M. and Ward, J. M.: Quantitative electron microscopic analysis of changes in peroxisomes and endoplasmic reticulum induced in mice during hepatocarcinogenesis by diethylnitrosamine promoted by di(2-ethylhexyl)phthalate or phenobarbital. J. Exp. Pathol. 1: 287-294, 1984.

Ward, J. M., Diwan, B. A., Ohshima, M., Hu, H., Schuller, H. M. and Rice, J. M.: Tumor initiating and promoting activities of di(2-ethylhexyl)phthalate in vivo and in vitro. Environ. Hlth. Perspect. (In Press)

Ward, J. M., Ohshima, M., Lynch, P. and Riggs, C.: Di(2-ethylhexyl)phthalate but not phenobarbital promotes n-nitrosodiethylamine-initiated hepatocellular proliferative lesions after short-term exposure in male B6C3F1 mice. Cancer Lett. 24: 49-55, 1984.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05350-03 LCC

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

The Thymic Microenvironment During T-Cell Lymphoma Development

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	U. I. Heine	Chief, Ultrastructural Studies Section	LCC	NCI
Other:	J. L. Junker	Staff Fellow	LCC	NCI

COOPERATING UNITS (if any)

Pathology Institute, University of Cologne, Cologne, Germany (G. R. F. Krueger); Biological Products Laboratory, Program Resources Inc., Frederick, MD (E. F. Munoz)

LAB/BRANCH

Laboratory of Comparative Carcinogenesis

SECTION

Ultrastructural Studies Section

INSTITUTE AND LOCATION

NCI, NIH, Frederick, Maryland 21701

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.0	0.5	0.5

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Maturation and proliferation of prethymic T-progenitor cells to mature lymphocytes depend on regulatory mechanisms in the thymus where the T-progenitors must interact with nonlymphoid, epithelial cells to be able to differentiate. The thymic epithelial cells provide a specific microenvironment capable of directing proliferation and maturation. It has been shown previously in mice that during the early phase of M-MuLV-induced lymphomagenesis, prethymic stem cells of the T-cell lineage derived from blood-forming tissues accumulate in the thymus where they encounter a differentiation block; subsequently, uncontrolled proliferation of the stem cells will lead to generalized lymphoma. The aim of this study is to elucidate the mechanism of the intrathymic differentiation block of prethymic lymphoid stem cells that gives rise to systemic malignant lymphoma of the Thy⁻ cell type. In vivo experiments, using the Moloney virus-induced lymphoma in the BALB/c mouse as a model, have been performed, and the phenotype and distribution of the major thymic cell populations have been characterized at different stages of tumorigenesis by light and electron microscopy. Immunofluorescence studies for the presence of thymopoietin II and serum thymus factor were carried out to determine the functional state of the epithelial cells of the thymus, which provide a microenvironment necessary for the differentiation of prethymic stem cells to lymphocytes of the T-lineage, are a prime target for transformation, as these cells undergo phenotypic changes, and are rendered functionally defective prior to lymphoma development. It is suggested that incompetent epithelial cells cause the progressive accumulation of nondifferentiating T-cell precursors producing a dysregulative lymphoma. Evaluation of the thymic microenvironment in mice of different strains (AKR, C3H, C57BL, BALB/c) expressing varying capabilities for the induction of lymphoma indicates that the changes described above may be of wider significance for lymphomagenesis.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

Ursula I. Heine	Chief, Ultrastructural Studies Section	LCC	NCI
James L. Junker	Staff Fellow	LCC	NCI

Objectives:

Our principal aim is to identify the role of the thymic microenvironment in T-cell lymphoma development. Special emphasis is directed towards the study of the thymic epithelial cells, as the latter may represent a critical target cell that by dysfunction initiates leukemogenesis.

Methods Employed:

Malignant lymphomas were induced by infecting newborn BALB/c HAN mice with 0.2 ml of Moloney murine leukemia virus suspension, 6.8 log FFU/ml. Infectivity studies were carried out at the University of Cologne, whereas the cell biology studies were pursued in this Laboratory. Thymic tissue, obtained from control and infected animals at biweekly intervals, was subjected to histological and cytological examinations using standard procedures for light, transmission and scanning electron microscopy. The quantitation of the cell population was performed on semithin sections in weekly intervals until tumor development. The functional state of the thymic epithelial cells was tested by identifying thymic hormone production by immunofluorescence using antisera against thymopoietin II and serum thymus factor. Comparative studies were carried out using mice of different origins, such as AKR, C3H, C57BL, and BALB/c.

Major Findings:

Current evidence shows that the thymic reticular epithelial cells have a major role in the maturation of prethymic stem cells of the T-cell lineage to functionally mature T-cells. Characterization of the reticular epithelial cells in the thymic cortex revealed, in control and infected animals the presence of two differentiation stages of these cells classifiable as an immature type, resembling the epithelial cell at the periphery of the thymus primordium, and a mature cell type. Quantitation of the different cells during lymphoma development indicates a shift in the cell population as the number of mature reticular epithelial cells declines early during leukemogenesis, coinciding with a progressive degeneration of these cells. These events coincide with an elevation in the number of immature epithelial cells and the prevalence of a new epithelial cell type of hyperchromatic appearance. Concomitantly, immunofluorescence studies for thymopoietin II and serum thymus factor show a marked decrease of these hormones only in epithelial cells of infected mice. The accumulation of lymphoblasts in the thymic cortex of infected animals, denoting lymphoma development, was found to be secondary to the shift in the population of the reticular epithelial cells. Our data, concerning the chronological sequence of increase in, as well as depletion of, the different cell types in the thymus during leukemogenesis, which coincides with the appearance of deficiencies in the hormonal competence of the reticular epithelial cells, suggest that a prime target for viral

infection is the thymic reticular epithelial cell. The data are in agreement for both the Moloney murine leukemia virus-induced lymphoma and the AKR lymphoma. They appear to be specific for lymphoma development in general as thymi of mice expressing a low capability of lymphoma development do not show such shifts in cell population during the life cycle of the animal.

Significance to Biomedical Research and the Program of the Institute:

One of the missions of the National Cancer Institute is the elucidation of the mechanisms leading to tumor production and, consequently, the establishment of protocols for treatment. Research of animal models of human lymphomas provides insight into such mechanisms. The aim of this study is to investigate changes in the thymic microenvironment that may be causative for the development of T-cell lymphomas.

Proposed Course:

This work was done in collaboration with the Institute of Pathology, University of Cologne. The project is now terminated.

Publications:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05351-03 LCC

PERIOD COVERED
October 1, 1984 to September 30, 1985

TITLE OF PROJECT (*80 characters or less. Title must fit on one line between the borders.*)
Ultrastructural Pathology: Morphological Analysis of Natural Killer Cell Activity

PRINCIPAL INVESTIGATOR (*List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	J. L. Junker	Staff Fellow	LCC	NCI
Others:	Y. Gong	Visiting Fellow	BTB	NCI
	J. M. Ward	Chief, Tumor Pathol. and Pathogen. Section	LCC	NCI
	C. Reynolds	Senior Staff Fellow	BTB	NCI

COOPERATING UNITS (*if any*)

None

LAB/BRANCH
Laboratory of Comparative Carcinogenesis

SECTION
Ultrastructural Studies Section

INSTITUTE AND LOCATION
NCI, NIH, Frederick, Maryland 21701

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1	1	0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK (*Use standard unreduced type. Do not exceed the space provided.*)

Studies involve collaboration on the characterization of neoplastic cells and the study of tumor histogenesis. The aim of the present investigation is to define, by high resolution and immunoelectron microscopy, changes in natural killer (NK) cells and targeted tumor cells which correlate with NK cell activity. Large granular lymphocytes have been identified with cytotoxic activity, probably via secretion of a cytolytic material (cytolysin) which is stored in cytoplasmic granules. Using recently produced antibodies (see project Z01CM09228-05 BTB) against cytolytic components, it has been possible to localize this cytolysin component to the cytoplasmic granules using colloidal gold probes on thin sections of glycol methacylate embedded tissue. It should now be possible to examine the movement of cytolytic proteins during NK cell attack of targeted tumorigenic cells.

PHS 6040 (Rev. 1/84)

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

J. L. Junker	Staff Fellow	LCC	NCI
Y. Gong	Visiting Fellow	BTB	NCI
J. M. Ward	Chief, Tumor Pathology and Pathogenesis Section	LCC	NCI
C. Reynolds	Senior Staff Fellow	BTB	NCI

Objectives:

To identify large granular lymphocytes (LGLs) in different tissues at high resolution by immunoelectron microscopy. To examine in vitro and in vivo the natural killer (NK) activity of LGLs on target cells, especially tumor cells induced by carcinogens. To study the role of LGL granules in cytolysis by examining the movements of secretion of these granules during the cytotoxic reaction.

Methods Employed:

Electron microscopic immunocytochemistry was performed using preembedding and post-embedding staining protocols. For preembedding staining, immunoperoxidase staining was performed using the avidin-biotin-peroxidase complex (ABC) method on cryostat sections of fixed tissue. For postembedding staining, LGLs isolated from rat blood were embedded in methacrylate and thin sections were stained using colloidal gold probes.

Major Findings:

Progress toward meeting our objectives has been made using antibodies to the cytoplasmic granules of NK cells (see project Z01CM09228-05 BTB). Staining of the granules in LGLs can be demonstrated on thin sections of glycol methacrylate embedded tissue using Protein A - colloidal gold to label the primary antibody. Inasmuch as such success at the ultrastructural level was not achieved using, as a marker for NK cells, OX-8 (an antibody which labels a cell surface antigen on NK cells and cytotoxic/suppressor T-lymphocytes), similar experiments using OX-8 have been suspended.

Significance to Biomedical Research and the Program of the Institute:

Lymphocyte cytotoxicity plays a major role in the immune response against foreign tissue, virally infected cells, and tumors. LGLs, a subpopulation of lymphocytes, have been identified with NK cell activity, and in vitro ultrastructural and biochemical studies have suggested a mechanism of target cell lysis involving the secretion of the contents of cytoplasmic granules (Millard, P. J., et al. J. Immunol. 132: 3197-3204, 1984). Antibodies to the contents of the cytolytic granules make it possible to study the process of NK cell activity structurally. The study of the interactions of NK and tumor cells will contribute to the understanding of natural defense mechanisms against cancer.

Proposed Course:

Experiments are currently underway to increase the sensitivity of the staining of the cytolytic granules, in order to be able to study the movement of cytolytic materials during NK cell attack on targeted tumor cells. Following the analysis of this process in vitro, these techniques will be applied to intact tissue in order to examine the physiological role of NK cell activity in tumor prevention.

Publications:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05352-03 LCC

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Metabolic and Pharmacological Determinants in Perinatal Carcinogenesis

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	L. M. Anderson	Expert	LCC NCI
Others:	J. M. Rice	Chief, Perinatal Carcinogenesis Section	LCC NCI
	J. M. Ward	Chief, Tumor Pathol. and Pathogen. Section	LCC NCI

COOPERATING UNITS (if any)

Program Resources, Inc., Frederick, MD (H. Issaq); University of South Florida, Tampa, FL (A. Giner-Sorolla)

LAB/BRANCH

Laboratory of Comparative Carcinogenesis

SECTION

Perinatal Carcinogenesis Section

INSTITUTE AND LOCATION

NCI, NIH, Frederick, Maryland 21701

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.25	0.5	0.75

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Sensitivity factors in perinatal carcinogenesis are studied through analysis of the roles of fetal and maternal metabolism of chemical carcinogens and of promotion later in life of tumors initiated during the perinatal period. In a pharmacogenetic investigation in mice of transplacental carcinogenesis by methylcholanthrene, genetic backcrosses of C57BL/6 and DBA/2 mice were employed to produce fetuses which were, in the same mother, either inducible or noninducible for the enzymes which metabolize this carcinogen. The mothers themselves were either inducible or non-inducible. Induction-responsive offspring exhibited 2-3 times more lung and liver tumors than did nonresponsive littermates. Furthermore, offspring of nonresponsive mothers developed 3-4 times more lung and liver tumors than did those of responsive mothers. Thus, the numbers of lung and liver tumors were greatest when the fetus was inducible and the mother noninducible. Important roles for both fetal and maternal metabolism are thus confirmed. In an assay of the nitroso derivative of a common pharmaceutical, N-nitrosocimetidine, given transplacentally, during lactation, and chronically, treatment with this chemical was associated with an increase in size and malignancy of lung tumors but a reduction in numbers of mammary tumors. Studies of the effects of this chemical on tumor development are ongoing. In a test of the effect of polychlorinated biphenyls (PCBs) on the development of tumors initiated in infant mice by dimethylnitrosamine, it was found that treatment with the PCBs 4 days after the carcinogen resulted in a doubling in the numbers of lung tumors at 2 endpoints in time, an effect associated with retention in the bodies of the mice of 2 specific hexachlorobiphenyl congeners. Thus, PCBs may result in tumor promotion in an extrahepatic organ, and may do so after a single treatment with the chemical. There were also multiple significant effects on the liver, including a reduction in number but an increase in size of neoplasms. This finding is of particular interest in light of the tendency of PCBs to accumulate in milk.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

L. M. Anderson	Expert	LCC	NCI
J. M. Rice	Chief, Perinatal Carcinogenesis Section	LCC	NCI
J. M. Ward	Chief, Tumor Pathology and Pathogenesis Section	LCC	NCI

Objectives:

To identify and characterize the cellular and organismal factors that determine the susceptibility of immature animals to carcinogens and that influence the development of tumors initiated during the perinatal period. Specific objectives for current projects are (1) determination of the role of maternal and fetal genotypes with regard to inducibility of aryl hydrocarbon metabolism in susceptibility to transplacental carcinogenesis by methylcholanthrene (MC); (2) assay of effects of nitrosocimetidine (NCM), a DNA-alkylating derivative of a widely-used pharmaceutical drug, on perinatal animals and on tumors; and (3) test of the effects of polychlorinated biphenyls (PCBs) on the development of tumors initiated in infant mice by dimethylnitrosamine. Experiments have recently been completed for each of these projects.

Methods Employed:

In the transplacental pharmacogenetics experiment, C57BL/6 mice (inducible for aryl hydrocarbon metabolism) and DBA/2 mice (noninducible) were used; in crosses and backcrosses of these strains, inducibility is inherited as a Mendelian dominant trait. (C57 x DBA)F_1 females mated to DBA males and DBA females mated with F_1 males were treated i.p. on the 17th day of gestation with one of several doses of MC or oil. Metabolic phenotyping of the progeny of these crosses was carried out at nine months of age by treatment with MC 48 hours prior to kill, followed by an isotopic assay for conversion of MC to primary oxidized and waterphase products by liver homogenates. The rest of each mouse was examined for tumors and the tumors confirmed by histopathology. Detailed statistical analysis of the data was performed.

In the assay of NCM, (C57BL/6 x BALB/c)F_1 mice were exposed to this agent in the drinking water at the usual human dose or at a tenfold greater dose, from two weeks preconception through fetal and neonatal development and the remainder of their lives. The precursors of NCM, cimetidine and sodium nitrite, were also administered to other groups of mice, singly and in combination. The mice were killed when moribund and examined for tumors.

In the test for the effects of PCBs, infant male Swiss mice were given a single treatment with dimethylnitrosamine, 5 mg/kg, i.p., on the fourth day of life. On the eighth day each received, by stomach tube, 50, 250, or 500 mg/kg of Aroclor 1254 (a commercial PCB mixture) in olive oil, or in oil alone. The mice received no further treatment and were killed at 16 or 28 weeks of age. Lungs, all liver tumors, and a portion of normal liver were fixed for histology, and the remainder of the livers and carcasses frozen for determination of PCB content. Lung and liver tumors and foci of hepatocellular proliferation were examined and measured. Body and liver

content of PCBs were quantified by extraction and gas chromatography with electron capture detection, with reference to standard dilutions of Aroclor 1254. Specific congeners were identified by reference to pure standards, with co-chromatography in 3 systems plus mass spectral analysis.

Major Findings:

In the transplacental pharmacogenetics experiment, the MC-exposed offspring which were responsive to induction of MC-metabolizing enzymes (genotype Ah^dAh^d) developed significantly more lung tumors than did nonresponsive littermates (Ah^bAh^d). This was true for both percent mice with tumor, especially at lower doses, and average number of tumors per mouse, especially at higher doses, and was observed for the progeny of both F_1 and DBA mothers. This difference was also noted for liver tumors in males and was most significant for the male offspring of the F_1 mothers. In addition, offspring of DBA mothers developed significantly more tumors of both lung and liver than did those of F_1 mothers given the same dose of MC, a distinction which was probably ascribable to inducibility of MC metabolism in F_1 but not DBA mothers. These results provide the first clear evidence that fetal metabolism of carcinogens is a determining factor in their susceptibility to tumorigenesis, and further point to an important protective role of maternal metabolism.

In the assay of NCM and its precursors, cimetidine, which is a drug commonly prescribed for ulcers, was tested for the first time in mice and for the first time transplacentally. The only significant effect was a small increase in incidence of lymphomas in females given cimetidine. No mammary tumors occurred in any female mouse given either dose of NCM, compared with a consistent incidence of 6-17% in all other groups. Treatment with the high dose of NCM or its combined precursors was associated with very large lung tumors in females and with metastatic lung tumors in males. Taken together, these results confirm lack of tumorigenicity of NCM but suggest effects on tumor development.

In the study of the effect of PCBs on the development of tumors initiated in infant mice by dimethylnitrosamine, the highest PCB dose, 500 mg/kg, resulted in a doubling of the incidence of lung tumors at both 16 and 28 weeks, differences of statistical significance. There were complex effects on the liver. Stimulatory effects included increased liver weight, degree of cytomegaly, and size of foci of hepatocellular proliferation when DMN was followed by PCBs, and sizes of liver tumors correlated positively with body content of PCBs after the 250 mg/kg dose. On the other hand, the PCBs caused a decrease in the number of liver foci and liver tumors. Reproductive capabilities of the mice were tested but no evidence of feminizing effects was found. The bodies and livers of the mice were found to contain mainly two congeners, 2,4,5,2',4',5'- and 2,4,5,3',4',5'-hexachlorobiphenyls, and the amounts were within the range of quantities found in human tissues.

Significance to Biomedical Research and the Program of the Institute:

Identification of the factors which contribute to fetal risk for tumorigenesis would clearly be useful for discerning perinatal humans at risk and for design of preventive measures. We have now established one such factor, at least for mice exposed transplacentally to a single dose of a polycyclic aromatic hydrocarbon carcinogen: genotype-determined responsiveness to induction of metabolism of this agent. The nature of the mother was also found to be important, with more tumors arising in

the offspring of the noninducible mother. After further study with animal models, considerations could begin of the applicability of this principle to human cancers, especially those of children and young adults.

The assay of NCM and its precursors was of practical importance, because of the widespread exposure of humans to the precursors and the likely formation of NCM in the stomach. Confirmation of lack of potent tumorigenicity of cimetidine and of nitrite, even when early life exposure was included, is reassuring. However, the small but significant increase in lymphomas in the females receiving cimetidine perhaps calls for further study. An effect of NCM on tumor development, if confirmed, will be of considerable interest as an example of an alkylating agent which is neither an initiator nor promoter of tumors, but stimulates progression.

With regard to the effect of PCBs on tumors initiated by dimethylnitrosamine, these findings are of potential public health importance because of the widespread occurrence of PCBs in human tissues. Our study revealed significant, complex effects of these chemicals on tumor development after a single exposure during infancy. If confirmed by more extensive studies with this and other model systems, the results could provide an important clue for epidemiological studies correlating human PCB exposure and body burden with cancer risk.

Proposed Course:

In the pharmacogenetic project, an experiment is in progress in which the same genetic crosses have been used, but with treatment of the mothers with the noncarcinogenic inducer beta-naphthoflavone preceding the MC. According to the results of our first experiment, this agent should increase the numbers of tumors initiated in the responsive fetuses, compared with nonresponsive littermates, but decrease the risk of the fetuses of F_1 mothers compared with DBAs. Experiments are planned with different strains, carcinogens, and routes of administration. Studies of carcinogen metabolism and DNA adduction in fetal tissues and of maternal/fetal carcinogen pharmacokinetics are also anticipated.

A test of NCM as a transplacental tumorigen is in progress. This agent is also being tested for its effects on development of skin tumors on Sencar mice, lung tumors in BALB/c mice, and mammary tumors in (C57BL/6 x C3H)F_1 mice in order to test the hypothesis that it can cause some tumors to progress to malignancy and/or have a chemotherapeutic effect.

The effects of PCBs as tumor promoters in mice will be further characterized. The hexachlorobiphenyl congeners that were found to be retained in the bodies of the mice will be tested individually. These and the complex mixture will be given to adult mice after DMN to determine whether the effects observed were specific to the perinatal period. Effects of lower doses of PCBs, down to 0.1 mg/kg, will be examined. Mechanisms of the effects of the PCBs will be studied, especially changes in steroid hormone metabolizing enzymes and species of cytochrome P450 in liver and lung. Bindings of ^{14}C-PCBs in the target organs will be investigated with regard to cellular and intracellular site and covalent modification of macromolecules.

An experiment is in progress to assess the effectiveness of nitrosamines as transplacental neurogenic carcinogens in C3H mice in an attempt to establish an animal model for perinatal causation of brain tumors by environmental nitroso compounds.

Publications:

Anderson, L.M., Donovan, P.J. and Rice, J.M.: Risk assessment for transplacental carcinogenesis. In Li, A.P., (Ed.): New Approaches in Toxicity Testing and Their Application to Human Risk Assessment. New York, Raven Press, 1985, pp. 179-202.

Anderson, L.M., Giner-Sorolla, A., Haller, I.M. and Budinger, J.M.: Effects of cimetidine, nitrite, cimetidine plus nitrite, and nitrosocimetidine on tumors in mice, following transplacental plus chronic lifetime exposure. Cancer Res. (In Press)

Anderson, L.M., Jones, A.B., Riggs, C.W. and Ohshima, M.: Fetal mouse susceptibility to transplacental lung and liver carcinogenesis by 3-methylcholanthrene: positive correlation with responsiveness to inducers of aromatic hydrocarbon metabolism. Carcinogenesis. (In Press)

Rice, J.M., Donovan, P.J. and Anderson, L.M.: Mutagenesis and carcinogenesis. In Fabro, S. and Scialli, A.R. (Eds.): Drug and Chemical Action in Pregnancy, New York, Marcel Dekker. (In Press)

Ward, J.M., Singh, G., Katyal, S.L., Anderson, L.M. and Kovatch, R.M.: Immunocytochemical localization of the surfactant apoprotein and Clara cell antigen in chemically induced and naturally occurring pulmonary neoplasms of mice. Amer. J. Pathol. 118: 493-499, 1985.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05353-03 LCC

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Sensitivity Factors in Special Carcinogenesis Models

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	L. M. Anderson	Expert	LCC	NCI
Others:	S. Saraswathy	Visiting Fellow	LCC	NCI
	J. M. Rice	Chief, Perinatal Carcinogenesis Section	LCC	NCI

COOPERATING UNITS (if any)

Temple University, Philadelphia, PA (G. Harrington and P. Magee)

LAB/BRANCH

Laboratory of Comparative Carcinogenesis

SECTION

Perinatal Carcinogenesis Section

INSTITUTE AND LOCATION

NCI, NIH, Frederick, Maryland 21701

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.25	1.0	0.25

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Two animal models have proved to be particularly promising for study of modulating processes in chemical carcinogenesis. In the first, athymic nude (nu/nu) mice and their normal, hairy counterparts (nu/+) were subjected to a variety of procedures known to result in tumor formation with normal mouse skin. The study revealed, for the first time, that nudes are more sensitive than normal to causation of squamous skin tumors. This was true for a variety of chemical and physical causative agents, including dimethylbenzanthracene (DMBA) as initiator and 12-O-tetradecanoylphorbol-13-acetate (TPA) as a promoter, repeated treatment with methylnitrosourea (MNU), a single dose of MNU followed by TPA, intraperitoneal ethylnitrosourea followed by TPA, or repeated exposure to ultraviolet light. These protocols may now be used to study the special properties of nude skin and/or lack of immune surveillance as determining factors in sensitivity to carcinogenesis. Furthermore, the DMBA treatment resulted in a high incidence of sebaceous adenomas, which were frequently overlaid by hyperplastic epidermis and sometimes squamous papillomas, providing a model for study of local cellular interactions in tumorigenesis. In the second project, the role of induction of enzymes metabolizing chemical carcinogens in susceptibility to tumorigenesis was investigated by use of 6 mouse strains of varying inducibility, including inbred C57BL/6, BALB/c, and C3H (inbred, highly inducible), noninbred Swiss (moderately inducible), and DBA and AKR (noninducible). Females of each strain were given the carcinogen; methylcholanthrene (MC), i.e., with or without prior exposure to the enzyme inducer beta-naphthoflavone (beta-NF). The beta-NF pretreatment protected against mortality due to MC-caused malignancy and specific tumor types, including those of lung, forestomach, mammae, mesothelium, and lymphoid and connective tissue by 50-100% in the induction-responsive strains, whereas in the DBA mice the only effect was a moderate reduction in lymphomas. There was no effect in the AKRs. This confirmation of the protective potential of induction of carcinogen detoxification pathways could be of potential public health usefulness.

PHS 6040 (Rev. 1/84)

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

L. M. Anderson	Expert	LCC	NCI
S. Saraswathy	Visiting Fellow	LCC	NCI
J. M. Rice	Chief, Perinatal Carcinogenesis Section	LCC	NCI

Objectives:

To investigate reasons for differing susceptibilities to carcinogenesis among individuals and between organs, by analytical application of animal tumorigenesis models with unique qualitative or quantitative sensitivity to chemical carcinogens. Two models have given especially fruitful results. An experiment with athymic nude (nu/nu) and hairy (nu/+) mice has addressed the question of whether nude mice are sensitive to skin carcinogenesis by topical treatment with tumorigenic agents, and, if so, whether they differ significantly from their normal hairy counterparts. The role of the induction of the enzymes which metabolize chemical carcinogens, as a component determining the effectiveness of these agents, has been investigated by use of six mouse strains which vary considerably with regard to this property from highly inducible to noninducible.

Methods Employed:

Athymic nu/nu and phenotypically normal nu/+ mice (BALB/c background) were maintained under pathogen-free conditions. Ten mice of each sex and genotype received the following treatments: ethylnitrosourea (ENU, 25 mg/kg, i.p. twice), followed by 100 ng TPA twice weekly; dimethylbenzanthracene (DMBA, 500 µg, once); DMBA (125 µg, once), followed by TPA twice weekly; methylnitrosourea (MNU, 20 µl of 1% solution, once); MNU (0.5%, µl weekly); MNU (1%, once), followed by TPA twice weekly; and UV light, 9 J/m^2/sec, 15 min, 3x/week. Controls received TPA or no treatment. The mice were killed when moribund or after 13 months. All skin nodules greater than 4 mm in diameter were examined histologically. The role of genotype-related inducibility of aryl hydrocarbon metabolism in tumorigenesis was investigated with C57BL/6, BALB/c, and C3H mice (highly inducible), noninbred NIH Swiss (moderately inducible), and DBA/2 and AKR (noninducible). These received methylcholanthrene (MC, 20 mg/kg, once per week for 12 weeks, i.g.) or MC preceded 24 hours earlier with the inducer beta-naphthoflavone (beta-NF, 150 mg/kg, i.p.). The mice were killed when moribund or after 13 months, and all tumors examined histologically.

Major Findings:

In the experiment with nude mice, all of the treatments were quite effective in eliciting skin tumors on the nudes, in spite of numerous reports to the contrary by other investigators. The single dose of MNU caused a total of 27 skin tumors on the nudes, but none on the nu/+ mice. Repeated treatment with MNU was quite effective, causing multiple skin carcinomas by 9 months and significantly more squamous tumors at the site of treatment on the nudes compared with the nu/+ mice.

Similarly, ENU/TPA and MNU/TPA resulted in significantly more tumors on the nudes, resulting in 25-40% tumor-bearers. DMBA/TPA was the most effective regimen, giving a multiplicity of tumors on all mice, and again, significantly more squamous tumors at the site of treatment on the nudes. The relatively low dose of UV light caused squamous tumors on the nudes only (40% with tumors on the ears). Thus, single and repeated treatments with a direct-acting alkylating agent, i.p. as well as topical initiation, and nitrosourea or polycyclic aromatic hydrocarbon initiation followed by TPA promotion, as well as physical carcinogenesis by UV light, all served to cause skin tumors on the nudes and to permit determination of a difference between the nudes and the normal mice. Furthermore, in all groups a significant number of sebaceous adenomas occurred on the skins, in addition to the squamous papillomas; these were the most common type of tumor after the single dose of DMBA. A common and interesting property of these sebaceous tumors was a tendency to provoke hyperplasia on the part of the overlying epidermis, ranging from slight to pronounced, and occasionally including a well-developed squamous papilloma. About 70% of all sebaceous adenomas showed this associated hyperplasia.

In the second project, the enzyme inducer beta-NF was found to be quite effective in protecting against tumorigenesis by MC in those mouse strains with genotypes permitting an induction response to beta-NF. Even though 50-100% of the induction-responsive C57BL/6, C3H, and BALB/c mice had died within 12 months after treatment with MC only, other mice of these strains which received beta-NF before the MC were completely protected from MC-caused mortality. The Swiss mice, which are moderately inducible, enjoyed about 70% protection when beta-NF preceded the MC. The same treatment led to only a 30% reduction in mortality in the noninducible DBAs, and had no effect in the noninducible AKRs. Incidences of specific tumor types were compared. It was found that the beta-NF significantly reduced the numbers of MC-caused lymphomas, sarcomas, skin tumors, and forestomach papillomas in the C57BL, sarcomas, mesotheliomas, and mammary carcinomas in C3H, mesotheliomas in BALB/c, sarcomas, skin tumor, lung tumors and forestomach papillomas in Swiss, and lymphomas in DBAs. The beta-NF did not result in an increase in incidence of any type of tumor in any strain. These results confirm that induction of the enzymes which metabolize a polycyclic aromatic hydrocarbon carcinogen generally provides protection against the chemical's effect, even though a result of such induction is increased rate of activation to proximate carcinogenic forms. Evidently the detoxification role predominates.

In an ongoing study of the effects of chronic administration of low levels of dimethylnitrosamine (DMN) to mice, in special models designed to characterize the tumorigenic effects of environmental levels of this agent, a test of daily administration of low levels of DMN to strain A mice is in progress. In addition, accumulation of DMN in the blood and in tissues as a function of DMN dose and concomitant administration of ethanol is being assessed, with sensitive determinations of the DMN being carried out by Dr. G. Harrington. The results thus far indicate that quite large amounts of DMN must be administered before any can be detected in the blood and tissues, a finding relevant to reports of circulating DMN in humans. Also, co-administration of ethanol with the DMN affects both toxicity and appearance of detectable levels and appears in general to be protective.

Significance to Biomedical Research and the Program of the Institute:

Our demonstration of higher than normal sensitivity of nude mouse skin to carcinogenesis by a variety of agents is the first report of such a phenomenon in this widely-used experiment-of-nature. Failure of several other investigators to accomplish this was apparently due to use of nonpathogen free nudes and inappropriate choices of doses. The protocols which were established in this study may now be used to sort out the contributions of skin and of immune system function to the specific sensitivity, topics of obvious relevance to human cancer of the skin and other organs. Furthermore, the revelation of sebaceous adenomas as a major category of skin tumors is of practical significance for interpretation of carcinogen-skin target cell interactions. Gross skin nodules in experiments involving bioassay represent at least two distinct pathogenetic pathways starting from different tissue components. Simple enumeration of these nodules can no longer be considered fully informative. Also, the association of epidermal hyperplasia and papillomas with a high percentage of the sebaceous tumors suggests that this may be a useful model system for study of localized tumor promotion by factors of endogenous origin, a subject of special interest in light of recent identification of certain oncogene products as growth factors and demonstration of activation of a cellular activated oncogene in mouse skin papillomas.

Our findings with regard to the effects of beta-NF on tumorigenesis by MC provide confirmation of the hypothesis that induction of the enzymes which metabolize chemical carcinogens may be a generally applicable procedure for protection against the effects of these agents. Indeed this appears to be one of the most generally valid principles of modulation of carcinogenesis yet described. From a public health point of view, it is a particularly attractive principle, since induction of the enzymes may be effected by normal dietary ingredients. Also, metabolic phenotyping of individuals to determine responsiveness to induction could provide a measure of degree of carcinogenic risk.

Investigation of the effects of chronic treatment with environmental levels of a carcinogen such as DMN, to which people are commonly exposed in low concentrations, could be of significance if full characterization of biological interactions and mechanisms of effect could be used to predict and intervene in human risk. Our ongoing study is aimed at extending such characterization. Interactions with other agents to which people are commonly exposed, such as ethanol, are clearly of interest in this context.

Proposed Course:

The next step in analysis of the reasons for nude mouse sensitivity in skin carcinogenesis will be dissection of special properties of the skin from effects of the immune system. Experiments involving skin grafting and/or neonatal thymectomy, followed by carcinogen treatment, should provide a clear indication of whether the high number of squamous tumors on the nude mice reflect a skin-specific property or one dependent on absence of thymus. With regard to the sebaceous adenoma-epidermis interaction, immunohistochemical techniques will be used to probe for elaboration of growth factors and oncogene-associated products by the sebaceous tumors. Effects of extracts of these tumors on epidermis will also be tried.

The study of the protective effect of enzyme inducers in carcinogenesis will be extended to other inducers and types of carcinogens, including nitrosamines and aflatoxins, and by co-treatment of mice with blockers of metabolism. Use of back-crossed or congenic mice will confirm that the effects of the inducer are in fact related to enzyme induction and not some other coincidental action. In an attempt to extend these findings to the human, a method for metabolic phenotyping of humans by means of cultured hair follicles will be tried and, if found useful, applied in a small pilot study, with a view to eventual application in cancer epidemiology.

The investigation of chronic effects of DMN with and without ethanol will be continued, with histopathology and enzymology added to clarify interpretation of the toxic effects of DMN and the apparent protective effects of ethanol. Eventually these findings will be used in the design of tumorigenesis protocols.

Publications:

Harrington, G.W., Pylypiw, H.M., Anderson, L.M. and Magee, P.N.: Possible artifactual formation of N-nitrosamines in liquid nitrogen. Cancer Res. Bull. 1: 11, 1984.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05399-02 LCC

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Oncogene Expression in Chemically Induced Tumors

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	J. M. Rice	Chief	LCC	NCI
Others:	A. O. Perantoni	Microbiologist	LCC	NCI
	M. Watatani	Visiting Fellow	LCC	NCI
	C. D. Reed	Sr. Health Services Officer	LCC	NCI
	J. M. Ward	Chief, Tumor Pathology and Pathogenesis Section	LCC	NCI

COOPERATING UNITS (if any)

Microbiological Assoc., Inc., Bethesda, MD (M. L. Wenk); Basic Research Program, Litton Bionetics, Inc., Frederick, MD (S. Sukumar, M. Barbacid); Program Resources, Inc., Frederick, MD (O. S. Weislow)

LAB/BRANCH

Laboratory of Comparative Carcinogenesis

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Frederick, Maryland 21701

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
3.0	2.5	0.5

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The expression of activated cellular oncogenes in chemically induced rat tumors and the relationship of oncogene expression to progression from the normal to the neoplastic phenotype are studied using 3T3 transfection and hybridization techniques and monoclonal antibodies directed against the specific oncogene products. Four types of tumors have been generated by single injection of F344 rats using direct-acting alkylating agents; renal mesenchymal tumors induced by methyl(methoxymethyl)nitrosamine (DMN-OMe), intestinal adenocarcinomas induced by methyl(acetoxymethyl)nitrosamine (DMN-OAc), hepatocellular carcinomas induced by intraportal injection of DMN-OAc followed by phenobarbital promotion, and gliomas induced by transplacental exposure to nitrosoethylurea (ENU). DNA purified from these tumors is utilized for 3T3 transfection assays, which are particularly effective for the detection of the ras oncogene, and in Southern blot hybridizations with available oncogene probes. Activated Ki-ras oncogenes have been identified by 3T3 cell transfection and hybridization techniques in 13 primary renal mesenchymal tumors and in one nasal cavity carcinoma induced by DMN-OMe. Tumor DNA positive for activated oncogenes will be evaluated to determine whether the oncogene is of the wild-type or a mutant by restriction mapping and sequencing. Depending upon availability of oncogene products, hybridoma technology will be applied for the production of monoclonal antibodies specific for the product, and generated monoclonals will be used to characterize oncogene expression in preneoplastic, early and late stages of tumor development in vivo by immunohistochemical procedures.

PHS 6040 (Rev. 1/84)

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

J. M. Rice	Chief	LCC	NCI
A. Perantoni	Microbiologist	LCC	NCI
M. Watatani	Visting Fellow	LCC	NCI
J. Ward	Chief, Tumor Pathology and Pathogenesis Section	LCC	NCI
C. Reed	Sr. Health Services Officer	LCC	NCI

Objectives:

To identify activated oncogene sequences in four specific types of chemically induced neoplastic rat tissues and to compare these observations with oncogene expression in normal, non-neoplastic tissues. To isolate and characterize the oncogene sequences found for mutant or wild-type alleles. To generate immunologic probes for available oncogene products and to apply these to detect expression of oncogenes at different stages of tumor development as well as during embryonic or fetal development.

Methods Employed:

Tumor generation: Four particular types of neoplasms in F344 rats are being induced to supply DNA for transfection experiments. These tumors include renal mesenchymal tumors induced by single neonatal i.p. injections of methyl(methoxymethyl)nitrosamine (DMN-OMe), intestinal adenocarcinomas induced by single i.p. injections of methyl-(acetoxymethyl)nitrosamine (DMN-OAc) of 5 week-old animals, hepatocellular carcinomas induced by single intraportal injections of DMN-OAc to 100-gram rats which were subjected to partial hepatectomies 21-24 hours previously and subsequently promoted with phenobarbital in the diet, and gliomas of the brain induced by single transplacental exposure to nitrosoethylurea (ENU) at 15 days gestation. Each of these alkylating agents has a short half-life. Predictable latency periods after the single instillation until the development of the desired tumor and documented histogenetic sequences are associated with the development of each of these experimental tumor types.

Transfection experiments: DNA is purified from the various tumors and appropriate control tissues by standard protocols, precipitated with calcium phosphate, and incubated with a clone of NIH/3T3 cells known to be readily transfectable. Oncogene-positive clones are isolated to provide a source of DNA for oncogene sequence analysis.

Oncogene screening: Available clonal oncogenes will be radiolabeled and hybridized with tumor DNAs that have been digested with an appropriate restriction enzyme, separated by gel electrophoresis, and transferred to nitrocellulose. Rat oligonucleotide sequences and specific ras oncogene sequences are identified by blotting techniques. Activated oncogenes will be sequenced to determine their similarity to the wild-type allele.

Oncogene expression: Depending upon oncogene product availability monoclonal antibodies will be developed specifically for that product and will be used in immunohistochemical procedures to characterize expression of the oncogene during different stages of histogenesis in these chemically induced tumors.

Major Findings:

In preliminary studies, DNA was extracted from three F344 rat renal mesenchymal tumors that had been induced by DMN-OMe and then maintained by serial transplantation in syngeneic hosts for approximately 20 generations. High molecular weight DNA was isolated from 2-3 g tumor tissue by pronase digestion, phenol and chloroform:isoamyl alcohol extractions, and ethanol precipitation. Transfection assays were performed using a clone of NIH/3T3 cells that exhibits a low frequency of spontaneous transformation. 3T3 cells (1.3-1.5 x 10^5) were seeded into 100 mm culture dishes and exposed to calcium-phosphate precipitated DNA (80 μg nucleic acid/dish) 24 hrs later. Following a 20-22 hr incubation with the precipitate, cultures were washed with medium and maintained for 28 days. At this time, cultures were evaluated for morphologically transformed colonies. These positive transfectants were distinguished from spontaneous transformants by several criteria: refractile character of cells when observed in a flat field; starry-shaped dense colonies; clumping of cells that have retracted from the culture dish; and criss-crossed outgrowth of cells in areas where clumping has occurred. Clumps in positive colonies were removed and dispersed either into culture medium or soft agar. Cells in these cultures were serially propagated to obtain sufficient biological material for subsequent DNA extraction and Southern blotting. DNA for blotting was extracted as outlined above and digested with three restriction enzymes: Hind III, Bam H1, and Eco R1. Fragments were separated by agarose gel electrophoresis, blotted onto nitrocellulose filters, and hybridized with probes for various ras genes and for repetitive DNA sequences of rat origin. A Ki-ras isolate was obtained from one of these three transplanted tumors, suggesting the likelihood of encountering this same activated oncogene in primary renal mesenchymal tumors.

For generation of these tumors, 50 F344 rat pups of each sex received a single i.p. injection of DMN-OAc, 4 mmole/kg within 48 hours of birth, a protocol based on results previously obtained in this Laboratory. From the age of 12 weeks, rats were palpated twice weekly for renal enlargement. Animals with palpable renal masses were killed, and 2-3 g samples of tumor tissue and of liver, brain, and contralateral kidney were frozen for DNA extraction. A sample of each tumor and of each control tissue was fixed in formalin for histologic examination.

DNA was isolated for transfection as before, and transformed 3T3 clones were probed for rat repetitive sequences. When these were found, the transfected clones were further probed for Ki-ras sequences. The first 25 tumors studied yielded 20 that were suitable for transfection experiments, and of those, 7 yielded transformed colonies of NIH/3T3 cells, all of which contained rat repetitive DNA sequences and an activated Ki-ras oncogene. There was no apparent tendency of the transfection positive tumors to cluster among tumors with shorter or longer latencies, and at least one transfection-positive tumor occurred in a rat of each sex. Typical renal tumors were mesenchymal neoplasms of the kind typical for this species (cf. Hard, G. C., in Pathology of Tumours in Laboratory Animals, Vol. I, Part 2, IARC, Lyon, 1976, pp. 73-101). To date, 13 such tumors have yielded DNA that has successfully transfected NIH/3T3 cells. In addition, one intracerebral extension of a nasal

cavity adenocarcinoma was subjected to the transfection/blotting protocols and, like the renal tumors, found to contain activated Ki-ras. No transfection occurred when DNA from control tissues of the same animals was used in the transfection protocols.

Tumors generated by other protocols are currently under study.

Significance to Biomedical Research and the Program of the Institute:

The increasingly numerous genetic determinants that confer a neoplastic transformed phenotype on cells in which they or transition mutants of these oncogenes are highly expressed, strongly suggests a connection between abnormal genetic expression, including perhaps chemically inducible mutation or other forms of genetic damage and the activity of certain growth factors in the evolution of at least some form of neoplasia. It is of great importance to establish what role specific oncogenes play in the evolution of tumors caused by known etiologic agents of human cancer, including both chemicals and radiation.

Proposed Course:

To explore systematically by means of highly specific monoclonal/polyclonal antibodies, the expression in experimental neoplasms of selected oncogenes, especially the family of 12th-codon mutant ras oncogenes that have been demonstrated in both human and animal neoplasms. Emphasis will be placed on distinguishing between expression at the earliest morphologically detectable stages of neoplastic proliferation (in which case the oncogene may be inferred to participate in neoplastic transformation) and acquisition of expression at a later stage of evolution of a tumor, as during an adenoma to carcinoma in situ transition or in metastases rather than primary neoplasms.

Publications:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05465-01 LCC

PERIOD COVERED
October 1, 1984 to September 30, 1985

TITLE OF PROJECT (*80 characters or less. Title must fit on one line between the borders.*)
The Regulatory Role of Retinoids on the Development of the Avian Embryo

PRINCIPAL INVESTIGATOR (*List other professional personnel below the Principal Investigator.*) (*Name, title, laboratory, and institute affiliation*)

PI:	U. I. Heine	Chief, Ultrastructural Studies Section	LCC	NCI
Others:	J. L. Junker	Staff Fellow	LCC	NCI
	A. B. Roberts	Research Chemist	LC	NCI
	M. B. Sporn	Chief	LC	NCI

COOPERATING UNITS (*if any*)

Biological Products Laboratory, Program Resources Inc., Frederick, MD
(E. F. Munoz)

LAB/BRANCH
Laboratory of Comparative Carcinogenesis

SECTION
Ultrastructural Studies Section

INSTITUTE AND LOCATION
NCI, NIH, Frederick, Maryland 21701

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.5	0.5	1.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (*Use standard unreduced type. Do not exceed the space provided.*)

The regulatory role of retinoids in growth and differentiation has been examined in vitro and in vivo by light and scanning electron microscopy using retinoid-deficient and control quail embryos between the 6-15 somite stage, as well as 2- and 2.5-day-old embryos. Retinoid deprivation during embryonal development causes abnormalities in organs of epithelial and mesenchymal origin, most dramatically preventing the formation of the extraembryonal circulatory system. Initial observations with early quail blastodermis incubated in vivo or cultured in vitro suggested that the earliest observable developmental defects in the retinoid-deficient embryo were localized in the heart region. Light microscopic and SEM studies indicate that in the deficient embryo the following sequences of events lead to vascular abnormalities: 1) if development of the heart is retarded before fusion of the two primitive endothelial heart tubes takes place, i.e., prior to the 7-somite stage, a cardia bifida forms; 2) if retardation manifests itself sometime later, a single enlarged ventricle develops in situ in verso. Heart development is known to proceed in anteroposterior direction, and the impact of retinoid deficiency was found to be most severe in the posterior region. Since during development the pacemaker region that regulates heart function is always located in the most posterior region of the heart, growth retardation in that area due to retinoid deficiency also resulted in severe reduction of heart beats. In all cases, the omphalomesenteric veins that normally extend caudad from the sinus venosus are not developed and the endocardium of the heart terminates as a blind pouch in the mid-ventral region of the animal. The results thus provide evidence for the failure to establish, at the level of the omphalomesenteric veins, a connection between the embryonal and extraembryonal circulatory system in the retinoid-deficient quail embryos.

PHS 6040 (Rev. 1/84)

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

Ursula I. Heine	Chief, Ultrastructural Studies Section	LCC	NCI
James L. Junker	Staff Fellow	LCC	NCI
Anita B. Roberts	Research Chemist	LC	NCI
Michael B. Sporn	Chief	LC	NCI

Objectives:

To investigate the specific developmental defects of retinoid-deficient quail embryos in order to understand the molecular mechanisms of retinoid action in modifying cell differentiation and regulating cell proliferation. Specific aims are the establishment of a model system, the identification of retinoid-dependent stages in this system, and a morphological analysis of the retinoid-dependent defects.

Methods Employed:

In collaboration with the Laboratory of Chemoprevention, a quail embryo system has been established in which flocks were maintained on a retinoid- and carotenoid-deficient diet. Retinoid-deficient and control embryos between the 5-15 somite stage, as well as older embryos were cultured in vitro or grown in vivo in the presence and absence of retinoids. Retinoid-deficient and control embryos were subjected to histological examination at different times during their development using standard procedures for light and scanning electron microscopy. The functional state of the heart was evaluated by monitoring the heart rate.

Major Findings:

The regulatory role of retinoids, substances comprising both the natural and synthetic analogs of retinol, in growth and differentiation affects tissues of different origins, evolving from epiderm or mesenchyme. In the chick embryo, retinoid deprivation most dramatically prevents the formation of the extraembryonal vascular system; yet, after early addition of retinoids, the deficient embryos are capable of resuming normal development (Thompson et al., Brit. J. Nutr. 23: 471, 1969). In vivo studies using the quail embryo model showed that the basis for the defect is the retardation of heart growth resulting in the failure of the primitive heart tubes to open, thus preventing the formation of omphalomesenteric veins normally connecting the embryonal with the extraembryonal circulatory system. Early manifestation of the retinoid-deficient defect may result also in formation of a cardia bifida, late manifestation in development of a single dilated ventricle in situ inverso. In contrast, the extraembryonal vascular system of blood islands is well developed. Heart function as shown by the rate of heart beat is reduced in deficient embryos. Our in vitro studies demonstrate similar defects in the development of the circulatory system by culture of normal 24-hr embryos on retinoiddeficient agar medium; conversely, normal development is observed upon culture of retinoid-deficient embryos on retinoid-containing agar medium. Likewise, addition of retinoids to the developing quail embryo in vivo resulted in the reversal of the developmental defects.

Significance to Biomedical Research and the Program of the Institute:

In this report we have shown the usefulness of the quail embryo model as a research tool since normal development can be restored in retinoid-deficient embryos by supplying retinoids as evidenced by the formation of omphalomesenteric veins and a functional circulatory system. This model system might have valuable applications in the study of molecular mechanisms whereby retinoids control differentiation and proliferation of mesenchymal cells. Moreover, our present understanding of oncogenes, as developmentally regulated normal cellular genes, would suggest that mechanisms operating in this embryo system would shed light on the regulatory effects of retinoids on transformation processes as well.

Proposed Course:

In collaboration with the Laboratory of Chemoprevention, NCI, we intend to pursue these studies at a more mechanistic level. In retinoid-deficient and control embryos the role of matrix proteins in differentiation will be examined by using tissue sections for fluorescent staining of antibodies against such proteins. We also hope to be able to use the techniques of in situ hybridization to examine the roles of growth factors and oncogenes in differentiation of specific tissues and cell types of the embryo.

Publication:

Sporn, M.B., Roberts, A.B., Heine, U.I., Roche, N.S., Munoz, E.F., Smith, J.M., Smith, K.L., Dalton, S., Shealy, Y.F. and Dawson, M.I.: Retinoids and differentiation of cells of mesenchymal origin. Dermatologia. (In Press)

CONTRACT IN SUPPORT OF PROJECT NUMBERS:

Z01CP04582-10 LCC
Z01CP05157-06 LCC
Z01CP05093-07 LCC
Z01CP05299-04 LCC
Z01CP05303-04 LCC
Z01CP05399-02 LCC

MICROBIOLOGICAL ASSOCIATES (N01-CP-41014)

Title: Non-SPF Rodent Holding Facility for the Laboratory of Comparative Carcinogenesis

Current Annual Level: $347,481

Man Years: 4

Objectives: The purpose of this contract is to provide support services for the Laboratory of Comparative Carcinogenesis for long-term holding, treatment and observation of rodents in carcinogenesis investigations emphasizing lifetime tumor induction in rodents and related activities. The contract is specifically utilized for conducting experiments that require species or strains of rodents not available from the Frederick Cancer Research Facility (FCRF) animal production area, since such animals cannot be introduced into the LCC animal research facilities at the FCRF.

Protocols are developed in collaboration with LCC investigators and approved by an LCC project officer. Protocols involve the preparation, handling and administration of chemical solutions to animals according to NCI guidelines for the safety of personnel; specifications for holding, treatment, and data collection (including gross pathology data) for mice, hamsters, rats and related species; administration of chemical carcinogens to animals by skin painting, gavage, parenteral injection or other routes; storage of labile animal diets, reagents, tissues or other materials under controlled temperature conditions; qualitative or quantitative analysis of carcinogen preparations or of tissues of carcinogen treated animals and other necessary details are provided by LCC investigators. All fixed tissue specimens from carcinogenesis studies are sent to FCRF for histology and evaluation by NCI pathologists.

Major Contributions: This contract has made possible research involving adminis tration of chemical carcinogens to laboratory animals that could not have been accomplished at FCRF, including studies on the effects of retinoids on naturally occurring tumors of ACI rats, ENU in congenic strains of mice varying in their expression of murine retroviruses (in collaboration with Dr. Janet Hartley, NIAID), and carcinogenesis in mongolian gerbils, which vary strikingly from other rodents in their response to chemical carcinogens.

Proposed Course: To continue for the duration of the current contract to provide support to the LCC for studies requiring long-term holding of rodent species and strains that cannot be accommodated at FCRF because they are not free of detectable potential rodent pathogens.

ANNUAL REPORT OF

THE LABORATORY OF EXPERIMENTAL CARCINOGENESIS

NATIONAL CANCER INSTITUTE

October 1, 1984 through September 30, 1985

The major goals of the Laboratory of Experimental Carcinogenesis (LEC) are to elucidate mechanism(s) of malignant transformation in human and animal cells by chemical carcinogens and other cancer causing agents; to determine critical cellular and genetic factors involved in initiation, promotion and progression of these transformed cells; and to apply, whenever possible, the knowledge obtained from these studies towards effective prevention of cancer in man. In order to obtain these goals, LEC plans, develops and conducts a research program that includes (1) identification and characterization of exogenous and endogenous factors controlling initiation, promotion and progression of chemically-induced tumors; (2) studies on the regulation of gene expression in normal and neoplastic human and animal cells; (3) definition of the mechanism by which modifiers of cellular differentiation may inhibit and/or promote the neoplastic process; and (4) characterization of the metabolic processing and mutagenic potential of both known and suspected carcinogenic aromatic amines.

The LEC seeks to accomplish the goals listed above by bringing together expertise in the diverse disciplines of cell biology, chemical and viral carcinogenesis, molecular biology, protein and nucleotide chemistry, and computer science. The central hypothesis of research conducted by this Laboratory states that the neoplastic process, whether caused by chemical or biological agents, will not be defined without an integrated multidisciplinary research effort.

The following changes in the organization of the Laboratory took place in FY 1985. Two new research groups were established under the Office of the Chief: The Image Processing and Computer Science Group and Two-Dimensional Gel Electrophoresis Research Group. The Cell Biology Section has also been reorganized. This reorganization includes: (1) phasing out of the current work on chemically-induced murine leukemias and hemopoietic cell biology; and (2) establishing a developmental cell biology program based on the transgenic mouse system.

The Laboratory is composed of four sections, each of which is charged with major responsibilities towards the goals set out for LEC. Due to the integrated and multidisciplinary approach towards the understanding of the neoplastic process, considerable interactions occur between the sections. These areas of interaction are listed in the individual project reports.

The two research groups recently established under the Office of the Chief have considerable interaction. The Image Processing and Computer Science Group is primarily focused on the development of the computer based analysis of two-dimensional gel electrophoretograms as well as on other computer programming that is related to ongoing research in the Laboratory. The Two-Dimensional Gel Electrophoresis Research Group is responsible for running, maintaining and developing the gel electrophoresis system, and for applying this technique in our research program. This group is also a focal point for coordinating several collaborative research projects that involve two-dimensional gel electrophoresis (2-D).

Image Analysis and Computer Science Group:

From its inception, the major objective of the Laboratory's computer facility has been to further expand and develop the 2-D gel analysis system in order to facilitate the use of this important research technique in the analysis of the neoplastic process.

Advances in the Computerized Analysis of Two-Dimensional Gels. The computer system, developed in this laboratory, for analyzing two dimensional gels has been dubbed ELSIE III. It has been distributed to several other laboratories in the United States and Europe. Some of the more significant advances in the past year are outlined below.

(1) Findspots. A new spot finding algorithm has been developed that combines both the thresholding method described by Vo et al. (Anal. Biochem. 112, 258, 1981) and the negative core detection method of Lemkin (Comp. Biomed. Res. 13, 571, 1981). First, the second derivative of the surface of the gel is calculated in both the X and Y directions using an elliptical least squares convolution template to both smooth the data and calculate the second derivative in one operation. Regions where the second derivative becomes negative, in both directions, identify one or more spots. These "negative cores" regions are next subjected to a thresholding operation that searches for additional peaks within them. This algorithm improves the accuracy of the spot finding programs without significantly increasing the number of false spots detected (about 1%).

(2) Data Organization. The data for each gel is kept in several files; e.g., one file contains the raw scan data, another the spot shapes, yet another the position and intensity information for each spot. In order to associate each data file with the proper 2-D gel and to keep the number of files stored in any directory at a minimum, a single, uniquely named subdirectory is created for each gel and the data files for the gel are stored in that subdirectory. All programs that reference a gel are given only the name of that gel (the subdirectory); the user need not be concerned with the structure and names of the various sub-files that describe the gel. It is the program's responsibility to access the proper files and do the appropriate bookkeeping. This organization also allows us to change the names and structures of data files in a manner invisible to the users of the facility. Finally, the structure allows one to match two gels located anywhere on the computer system simply by specifying the complete path names of the two gel directories. A special file is created that incorporates the pathnames of the gels and the sets of spots matched between the gels.

(3) Analysis Tools. Several program "tools" have been developed to aid in the analysis of processed data. A brief outline of some of the more significant programs follows: (a) Desaturate combines information about heavy and light exposures of a gel. A new set of files is created for the gel in which "saturated" data in the heavy exposure is replaced by the "unsaturated" information in the light. This effectively extends the dynamic range of the gels.

(b) Manypairs matches together unmatched gels among a set of gels where some of them have been matched. For example, if we have four gels, A, B, C and D, where gel A has been matched to gels B, C and D, manypairs can be used to automatically match gel pairs B-C, B-D, and C-D.

(c) Plotcpm plots the intensity of groups of spots as a function of time for gels in an experiment. It can thus be used to plot the kinetics of synthesis of many individual proteins during the course of an experiment.

(d) Findquant finds spots that are statistically different (T test) in intensity between two or more sets of gels.

(e) Findqual uses statistical tests to find spots that are different qualitatively between two or more sets of gels; that is, spots that are intense in at least one set of gels and absent in at least one other.

(4) Measures: picking what to measure to identify unknowns. One way to identify an unknown item is to measure its traits and compare the measurements with those of items the unknown might be. However, it may not be necessary to measure all of the unknown's traits in order to identify the unknown. For example, if a protein were known to be listed in the Protein Sequence Database (PSD -- Georgetown University Medical Center), it is often possible to identify the protein by measuring only some of its 20 amino acids.

Measures is a program which will take known measurements to find the lowest-cost sets of traits to measure which, for learning which of the known times an unknown might be, are as good as measuring all the traits. By labeling cells with different amino acids and using ELSIE III to measure the relative incorporation of label into different polypeptides, it may be possible to simultaneously identify a large number of the proteins resolvable on the gels. The measures program indicates that, depending on the error involved, it should be possible to identify proteins on these gels by labeling with between 12 and 16 different amino acids.

The research in the Two-Dimensional Gel Electrophoresis Research Group is focused on two major projects:

A. Analysis of Polypeptide Changes during Cellular Differentiation and Transformation

This project combines studies in several model systems both within LEC and in collaboration with other research groups:

1) In the cultured mouse epidermal cell system (cultured keratinocytes) Ca^{+2} is a critical regulator of growth and differentiation. In certain cell types, a synergism exists between 12-O-tetradecanoylphorbol-13-acetate (TPA) and Ca^{+2} in the stimulation of proliferation. Ca^{+2} induced differentiation of keratinocytes is enhanced by concurrent treatment with TPA. Structural-activity studies indicate that receptor binding of phorbol esters (i.e. TPA) is required for induction of epidermal differentiation. Thus phorbol ester receptor (protein kinase C) may be a regulator of keratinocyte differentiation. If Ca^{+2} and TPA induce epidermal differentiation via a common pathway (i.e. involving protein kinase C), one would expect certain common patterns of polypeptide expression and phosphorylation following treatment with these inducers.

Mouse epidermal cells were cultured in the presence of either low (0.02-0.09 mM) or high (1.2 mM) Ca^{+2} and then treated with the TPA (0.01 and 0.1 μg/ml) for 1, 4, and 24 hours. Cells were then pulse-labeled with [14C] amino acids for 4 hours (except TPA treated cells which were labeled for only 1 hour). Two dimensional electrophoretic analysis of total cellular polypeptides from epidermal

cells grown in the presence of either low calcium (mainly basal cells) or high calcium (mainly keratinocytes) revealed only quantitative polypeptide differences. From 600-1200 polypeptides were visible on 2-D gels over the pH range 4.9-7.3 and molecular weight range of 15-130 kDa. The pattern of polypeptide synthesis 1-4 hours after TPA treatment in low Ca^{+2} medium was compared to the pattern of untreated cells or those shifted to high Ca^{+2} medium. TPA treatment effected the synthesis of 122 polypeptides by two-fold or more within one hour compared to untreated low Ca^{+2} controls; 1.4 mM Ca^{+2} resulted in a two-fold or more change in 63 polypeptides compared to untreated low Ca^{+2} controls. Of the polypeptides which were modulated, the rate of synthesis of 11 were altered by both Ca^{+2} and TPA; 7 were up-regulated and 4 were down-regulated, with changes in the same direction for both differentiating agents. This result suggests that a common program of protein synthesis is induced by both Ca^{+2} and TPA, and these proteins (polypeptides) are probably related to epidermal differentiation. One polypeptide (pI 6.0/MW 80 kDa) was increased (6-8 fold) by both Ca^{+2} and TPA.

In a preliminary phosphorylation experiment, [^{32}P]-labelled polypeptides were compared after TPA treatment (30 min) or exposure to 1.4 mM Ca^{+2}. Prior to 2-D electrophoresis, cells were fractionated into crude membrane and cytosolic preparations. No new or unique phosphorylated polypeptides were observed after either Ca^{+2} or TPA. Keratin polypeptides, present only in the membrane preparations, were heavily phosphorylated in low Ca^{+2} medium, but the level or pattern was not altered significantly by Ca^{+2} or TPA treatment. The phosphorylation of four polypeptides was increased by TPA and one of these was also increased by Ca^{+2}. The phosphorylation of two 30 kDa polypeptides with pIs of 5.8 and 6.0 (which may represent the same polypeptide with different degrees of phosphorylation) was increased 2-3 fold by TPA but only slightly by Ca^{+2}. Phosphorylation of a 42 kDa membrane polypeptide with a pI of 5.3 was increased 2-3 fold by both TPA and Ca^{+2}. The phosphorylation of a 73 kDa polypeptide was unaffected by Ca^{+2} but increased by TPA in membrane fractions. The degree of increased phosphorylation of this polypeptide is probably an underestimate, since increases in the level of phosphorylation of a group of 73 kDa polypeptides at pIs 5.75, 5.85, and 5.90 were also observed in the cytosolic fraction. This polypeptide and the two 30 kDa polypeptides were observed in both membrane and cytosolic fractions.

2) Normal human bronchial epithelial cells (NHBE) can be induced to undergo terminal squamous differentiation (including an increase in cell surface area, formation of cross-linked envelopes, and cessation of cell division) in the presence of TPA (3 nM) or certain blood derived serum (BDS) growth factors. In contrast, however, various lung carcinoma cell lines and Harvey v-ras DNA transfected NHBE (TBE-1 cells) are resistant to induction of terminal squamous differentiation by either BDS or 100 nM TPA. These results and the work of others suggest that an aberrant control of normal differentiation is positively correlated with malignant transformation (i.e. malignant cells have a reduced capacity to respond to factors which induce terminal differentiation in normal cells).

2-D electrophoresis of [^{14}C]-labeled polypeptides from NHBE, TBE-1, and the progressively more malignant lines, TBE-1SA (TBE-1 cells selected for anchorage-independent growth) and TBE-1SAT (cells derived from TBE-1SA tumors) revealed very similar polypeptide patterns although both qualitative and quantitative polypeptide differences were observed among the various cell lines. Comparison

of polypeptide patterns from normal NHBE and TBE-1 cells revealed only one qualitative difference. In TBE-1 a group of four polypeptides (probably glycosylated variants) (pI 5.5-5.7/MW 56-55 kDa) appeared. In control NHBE cells one minor polypeptide occurred at pI 5.7/55 kDa. Minor quantitative differences were also noted. No qualitative polypeptide differences were noted between TBE-1 and TBE-1SA although there were marked differences in the expression of five polypeptides: three polypeptides (pI 6.5/41 kDa; 6.6/40 kDa; and 6.7/39 kDa) were greatly increased while two polypeptides (5.6/40 kDa and 5.7/40 kDa) were decreased in TBE-1SA as compared to TBE-1 cells. Comparison of TBE-1SAT cells with TBE-1 cells showed at least three qualitative polypeptide differences and more numerous quantitative changes. New (or at least modified) polypeptides (pI 4.9/55 kDa; 6.4/66 kDa; and 6.25/58 kDa) were identified. Most prominent quantitative changes include marked increases in the expression of two polypeptides (pI 5.2/30 kDa and 6.9/26 kDa) and almost complete loss of expression of polypeptides (pI 5.6/40 kDa and 5.7/40 kDa).

3) The Syrian hamster fetal cell (HFC) offers a very attractive model for the _in vitro_ study of transformation (neoplastic and malignant) by a wide variety of chemical and nonchemical agents. At neutral pH, sodium bisulfite ($NaHSO_3$) induces dose dependent morphological transformation of HFC in the absence of detectable DNA damage. These bisulfite transformed lines formed anchorage independent colonies in soft agar and produced progressively growing fibrosarcomas in nude mice. 2-D analysis of [^{14}C] labeled polypeptides from $NaHSO_3$ induced transformed cells showed both qualitative and quantitative polypeptide differences. Seven neoplastic lines (A, D, E, F, G, H, I) had the same qualitative changes: polypeptides 1 and 2 (pI 5.1/52 kDa and 5.1/28 kDa) were shifted slightly to the acidic side; polypeptides 3 (6.8/44 kDa) and 4 (5.5/46 kDa) were new (except in line A); and polypeptide 5 (6.0/55 kDa) was missing. None of these changes were observed either 0 or 48 hours after $NaHSO_3$ treatment (10 μg/ml). The transformed $NaHSO_3$ lines differed quantitatively from untreated HFC in that 10-25% and 2-4% of the polypeptides exhibited differences in expression greater than two- and fourfold, respectively. Furthermore, there were 21 specific polypeptides with coordinate quantitative changes in all transformed lines. Eleven polypeptides were down-regulated while 10 were upregulated. These polypeptides were located throughout the gel with respect to MW and pI. However, six of these polypeptides were consistently localized in areas where the tropomyosins (pI 5.0-5.8/32-38 kDa) and actin (pI 6.1/45 kDA) are located.

Line A (20 to 25 population doublings after colony isolation) induced a tumor in only 1 of 12 nu/nu mice. The other six lines induced tumors in a minimum of 50% of the injected mice. After recloning in soft agarose, the subline A1 (MBSBA1) induced tumors in 100% of the injected mice. In lines A, A1, and TA1 (tumor derived) polypeptides 1 and 2 were shifted, polypeptide 3 was present, and polypeptide 5 was missing. Polypeptide 4 was found in lines A1 and TA1 but not in A. Therefore, the expression of polypeptide 4 appears to be associated with increased tumorgenicity.

Early polypeptide changes were determined in a series of 20 $NaHSO_3$ induced morphologic transformed colonies two days after isolation. Polypeptides 1 and 2 were always shifted and polypeptide 5 was missing. Polypeptide 3 was present in 7 of of the colonies, and polypeptide 4 was present in 14 of the clones. Some clones had neither 3 nor 4, both 3 and 4, or only 3 or 4. Thus polypeptide changes in 1, 2, and 5 are associated with early steps in the transformation process and

related to morpholgic changes, whereas polypeptides 3 and 4 appear to occur later and are more closely associated with acquisition of tumorigenicity.

B. Early Events in Chemically-Induced Rat Hepatocarcinogenesis

Chemically-induced rat hepatocarcinogenesis is the major experimental model system presently being used in the Laboratory to study the mechanism of neoplastic evolution. The application of the 2-D system has been a major part of this research effort:

1) Previous biochemical and histochemical studies have demonstrated that the levels or enzymic activities of numerous cytosolic proteins, such as alpha-feto-protein (AFP), albumin (Alb), DT-diaphorase (DT), aldehyde-NAD(P) oxido-reductase (aldehyde reductase; AlDH) and various glutathione-S-transferases (GST) as well as the membrane-associated proteins, gamma-glutamyl transpeptidase (GGT), epoxide hydrolase, and the cytochrome P-450 monooxygenases show marked alterations during hepatocarcinogenesis. Initial work was therefore directed towards establishing that 2-D electrophoresis could be used to reliably detect and quantitate changes in these known polypeptide markers during hepatocarcinogenesis.

Albumin (Alb) and AlDH were readily separated on 2-D gels using standard O'Farrell conditions (equilibrium isoelectric focusing in the first dimension 13,000 v-hrs). Identification of Alb and AlDH was verified by comigration with highly purified protein preparations and immunoblot analysis (Western transfers) with rabbit antirat antibody preparations to Alb and HTC hepatoma purified AlDH. Previous work has shown that albumin levels are markedly reduced in hepatocellular carcinomas; however, no significant differences in the expression of Alb (pI 6.6/66 kDa) were observed in cytosolic preparations from untreated control liver (0.89 + 0.12% of total integrated density of gel), preneoplastic or early neoplastic nodules. Although purified hepatoma derived AlDH separates as a single homogenous band (54 kDa) in 1D (SDS-PAGE) electrophoresis, 2-D electrophoresis separates AlDH as a group of 5 polypeptides with pI values of 6.8-7.1. No differences in the expression of AlDH were observed between preneoplastic nodules and neoplastic nodules; however, polypeptide (b) (pI 6.9), whose constitutive levels in untreated control cells is very low, appears to be shifted slightly toward the basic region in untreated control gels as compared to both preneoplastic and neoplastic nodules.

Initial attempts to identify either DT-diaphorase (DT) or the individual subunits of glutathione-S-transferase (GST) using the standard O'Farrell conditions were not successful due to the basic nature of the subunit polypeptides. DT and the individual Yc (28.5 kDa), Yb (27.5 kDa), and Ya (26 kDa) subunits of GST-A (YbYb), GST-B (YaYc), and ligandin (YaYa), as well as the recently described Yp (26 kDa) subunit of the placental form of GST (GST-P) were, however, separated on 2-D gels under non-equilibrium isoelectric focusing conditions (6000 v-hrs). DT and the subunits of GST were identified by comigration with highly purified enzyme preparations, immunoblot analysis, and comparison with published 2-D electrophoretic patterns of GST-A, GST-B, and GST-P. Marked increases in the expression of the Ya subunit (2-fold) of GST-B (and/or ligandin), the Yb subunit (4-fold) of GST-B and the three isoelectric point variants (Yp) (15-fold) of GST-P were observed in both preneoplastic and neoplastic nodules as compared to untreated control liver. No significant changes in the expression of the Yc subunit of GST-B were observed. The expression of DT-diaphorase (32 kDa) was also increased (2-3 fold) in both preneoplastic and neoplastic nodules as compared to control liver.

Attempts to localize the membrane associated GGT, one of the most reliable markers for preneoplastic liver transformation, from crude membrane preparations from preneoplastic and neoplastic nodules on 2-D gels using immunoblot analysis with antibody preparations to rat kidney GGT were not successful. Two-dimensional electrophoresis of purified rat kidney GGT showed that GGT is composed of two subunit chains consisting of at least 18 individual subunit polypeptides. Seven polypeptides (pI 7.0-5.4/23-26 kDa) comprise the light chain and 11 polypeptides (pI 7.1-5.8/51-53 kDa) comprise the heavy chain. Western blot analysis showed that all of these components were immunoreactive with a mixture of the two antibodies raised separately against the light and heavy subunit chains. Comparison of 2-D patterns from partially purified GGT from hyperplastic nodules revealed almost identical patterns to the 2-D patterns from purified rat kidney GGT. GGT purified from hyperplastic liver nodules was immunoreactive with antibody (both light and heavy chain) prepared against kidney GGT.

Work is currently in progress toward the localization of epoxide hydrolase and the various cytochrome P-450 isozymes. Due to their basic nature and high lipophilicity it will be necessary to use non-equilibrium isoelectric focusing in the first dimension. Resolution of the various cytochrome P-450 isozymes will be accomplished using immunoblot analysis with various antibody preparations to individually purified cytochrome P-450 species.

2) During the course of hepatocarcinogenesis one of the most important processes in the neoplastic transformation of the liver is the development of focal lesions of proliferative hepatocytes shortly after initiation. After further carcinogen treatment or after promotion, these lesions enlarge to form grossly visible hyperplastic nodules. These nodules have two options: the majority (90-98%) "redifferentiate" back to normal appearing liver while a few persist, enlarge further, and may serve as sites for the formation of the ultimate hepatocellular carcinomas. Since the hyperplastic nodule serves as a critical point in the formation of cancer we have recently begun a study concerning the biochemical nature of these nodules. Male Fischer rats were subjected to the standard Solt-Farber procedure for the induction of the formation of liver hyperplastic nodules. Individual nodules of approximately the same size (3-5 mm) were removed and a small section of each nodule was taken for histological staining (hematoxylin and eosin, GGT, glucose-6-phosphatase). Nodules were classified as being either preneoplastic or neoplastic on the basis of histological examination. All nodules, both preneoplastic and neoplastic, stained strongly for GGT activity. Two-dimensional electrophoretic separation of silver stained polypeptides from normal untreated rat liver tissue and from a neoplastic hyperplastic nodule were very similar although numerous qualitative and quantitiative polypeptide differences were readily detected. Approximately 1100-1200 polypeptides were readily visible on each electrophoretogram. To aid in analysis tissue samples (untreated control liver, preneoplastic, and neoplastic nodules) were fractionated into cytosolic and crude membrane preparations prior to 2-D analysis. Approximately 1000-1100 membrane and 800-1000 cytosolic polypeptides were readily resolved on each electrophoretogram. During hepatocarcinogenesis one cytosolic (A, pI 6.8/57 kDa) and three membrane-associated polypeptides (B, 6.25/41 kDa; C, 6.75/26 kDa; D, 6.05/21 kDa) were expressed in both preneoplastic and neoplastic nodules but not in untreated control liver samples. Quantitatively, polypeptides A, B, C, and D represented 0.11-0.12%, 0.16-0.14%, 0.25-0.28%, and 0.07-0.08%, respectively, of the total integrated density of the respective preneoplastic and neoplastic nodules. For comparison, actin, one of the major membrane associated, and albumin, one of the major cytosolic polypeptides were expressed at concentrations

of 1% and 1.5%, respectively. No qualitative spot differences (either in cytosolic or membrane fractions) were observed among preneoplastic nodules and/or neoplastic nodules themselves. Although only four qualitative polypeptide differences were observed during hepatocarcinogenesis numerous quantitative polypeptide differences were also detected in both preneoplastic and neoplastic nodules. Quantitative comparisons of polypeptides within the same type of tissues (e.g. untreated vs untreated; neoplastic vs neoplastic; or preneoplastic vs preneoplastic) revealed a relatively tight quantitative correspondence between paired spots in each of the same tissue types in both cytosolic and membrane fractions with correlation coefficients of 0.7-0.9. Greater scattering (correlation coefficients 0.5-0.6) of polypeptide densities were observed, however, when normal untreated liver samples were compared to either preneoplastic or neoplastic nodules. Comparison of polypeptides from two untreated control liver samples revealed that only 1% of the membrane (975 polypeptides compared) and 2.7% of the cytosolic (800 compared) showed quantitative variation greater than four-fold. Examination of the location of the polypeptides showing significant differences showed that many of these were located in regions of the electrophoretograms which are routinely highly variable (i.e. pH > 7.3 MW > 100 kDa). Comparison of 750-1000 membrane and 500-800 cytosolic polypeptides from preneoplastic and neoplastic nodules revealed that roughly 4-8% of the membrane and 6-10% of the cytosolic polypeptides were undergoing quantitative changes of at least four-fold during hepatocarcinogenesis. Polypeptides which showed significant modulation occurred at all pH and molecular weight regions. Twenty-one membrane-associated and 10 cytosolic polypeptides were down-regulated, while 14 membrane and 6 cytosolic polypeptides were up-regulated during hepatocarcinogenesis. In all but three polypeptides, the direction and magnitude of change were the same in both preneoplastic and neoplastic nodules.

3) One of the main characteristics of cancer cells is their marked heterogenicity with respect to cellular structure, biochemistry, immunology, etc., and one hypothesis states that this heterogenicity appears early in the carcinogenic process, possibly during initiation. Others feel, however, that this heterogenicity occurs only late in the process. In an attempt to address this problem, we have begun to investigate the heterogenicity/homogenicity of polypeptide expression in preneoplastic and neoplastic nodules isolated from different animals undergoing hepatocarcinogenesis. Hyperplastic nodules were induced in male Fischer rats (Solt-Farber technique) and 6 hours prior to sacrifice and isolation of hyperplastic nodules animals were treated (i.v. tail vein) with [^{35}S]-methionine (1 mCi). Untreated control animals were treated the same. Nodules were dissected, sections histologically scored as either preneoplastic or neoplastic, and the 2-D electrophoretic analysis of the cytosolic and crude membrane polypeptides performed. At least 20 nodules were analyzed from each animal. No significant differences in the incorporation of [^{35}S]-methionine into either membrane-associated or cytosolic polypeptides were observed between preneoplastic and neoplastic nodules and untreated control rat liver. Electrophoretograms were both silver stained to analyze for differences in constitutive polypeptide levels and then subjected to fluorography to analyze for differences in turnover rates of the individual polypeptides. Preliminary data obtained from the analysis of silver stained gels revealed marked homogenicity among the individual nodules isolated from separate animals. We are currently analyzing the [^{35}S] labeled polypeptides for differences in turnover rates for the individual polypeptides among the various nodules.

4) Work has been initiated towards the construction of a "liver-polypeptide" map. We have begun a systematic study dealing with the subcellular isolation of the various liver fractions (e.g., nuclei, mitochondria, plasma membranes, nuclear membranes, cytosolic proteins, gap proteins, etc.) and the 2-D electrophoretic analysis of their constitutive polypeptides. Once constructed this map will allow one to focus more closely on those polypeptides which are critically involved in the carcinogenic process(es).

The Chemical Carcinogenesis Section plans and develops laboratory research aimed at elucidating the mechanism(s) of malignant transformation in human and animal cells by chemical carcinogens and other cancer causing agents. The major efforts of the Section are focused on (1) identification and characterization of exogenous and endogenous factors controlling initiation, promotion and progression in chemically-induced murine hepatomas and human B-cell lymphomas; (2) applying advanced quantitative two-dimensional gel electrophoresis techniques of total cellular proteins to study protein changes during oncogenesis and to identify gene product(s) that are associated with the malignant transformation; (3) studying the metabolic processing and mutagenic potential of both known and suspected carcinogenic aromatic amines; and (4) definition of the mechanism by which modifiers of cellular differentiation may inhibit and/or promote the neoplastic process.

The following research projects are currently ongoing in the Section:

1. Chemical Carcinogenesis; In vitro Transformation with Viral Oncogenes; Tumor Progression

We have used a normal hepatocyte cell line derived from neonatal Fischer rats (FNRL) for transfection with plasmid DNA. The transfection method of Huang et al. (Cell 27: 245, 1981), with slight modifications, was employed in these experiments. The results so far obtained include: (1) the FNRL cells were transfected with pCneo10 plasmid conferring resistance to G418 (neomycin). The number of G418 resistant colonies per 100 ng of the pCneo10 plasmid was 6 for the FNRL cells, whereas 22 G418 resistant colonies per 100 ng of the DNA were observed in the 3T3 cells; (2) cotransfection of the FNRL cells with the neo-containing plasmid and MMTV-v-ras chimera and selection with G418 resistant colonies. These colonies are currently being characterized.

2. Regulation of Gene Expression and Differentiation in Neoplasia

A. Studies on the Human Promyelocytic Leukemia Cell Line HL60: Independent Expression of c-myc and N-ras during Growth and Differentiation. The HL60 cell line, a continuously proliferating suspension cell culture originally derived from a patient with acute promyelocytic leukemia, is widely used as an in vitro model for studying cellular differentiation along the myeloid/monocyte pathways. Treatment of HL60 cells with compounds such as retinoic acid, hypoxanthine, actinomycin D, butyrate, dimethyl sulfoxide and hexamethylene bisacetamide results in a greater than 90% commitment of the cells to differentiate, both functionally and morphologically, into mature granulocytes. Exposure of HL60 cells to phorbol esters, such as 12-O-tetradecanoylphorbol-13-acetate (TPA), results in an irreversible commitment to monocyte differentiation, characterized by the shift from suspension to adherent cells, the acquisition of macrophage associated surface markers, monocyte specific esterases and the cessation of cell growth associated with terminal differentiation.

Treatment with HL60 cells with difluoromethyl ornithine (DFMO), an irreversible inhibitor of ornithine decarboxylase (ODC), results in a cessation of growth without committing the cells to differentiate. However, DFMO does not prevent differentiation of HL60 cells into monocytes when TPA is subsequently added, thus demonstrating that TPA-induced monocytic differentiation is independent of the decrease in cell proliferation also associated with TPA treatment.

We investigated the expression of two of the HL60 associated oncogenes, c-myc and N-ras, both during terminal differentiation and growth inhibition by DFMO that is independent of terminal differentiation. The c-myc oncogene is present in multiple copies in this cell line as well as in the original cell isolate and is highly responsive to treatment with retinoic acid of dimethylsulfoxide. C-myc transcripts are reduced 80-90% in HL60 cells treated with these granulocyte differentiating compounds when compared to untreated controls. The N-ras oncogene has been reported to be the transformation specific gene in HL60 as assayed by the transfection of NIH3T3 cells.

We have associated the 8-10-fold decrease in c-myc transcripts with differentiation of the promyelocytes into mature monocytes. C-myc regulation also appears to be an early event of HL60 cells as evidenced by a burst of c-myc synthesis within the first hour of TPA treatment, followed by the rapid decline in the level of c-myc specific transcripts. This response was similar to that observed in mitogen stimulated B cells, T cells, and normal fibroblasts although the HL60 induced level was neither as dramatic nor as long as that reported for normal cells. Although this response was reportedly associated with the cell cycle regulation of proliferation in normal cells, the level of c-myc specific trancripts was found invariant during the HL60 cell cycle. In addition, unlike the mitogenic response of other agents, or normal resting cells, TPA induced differentiation of HL60 cells resulted in no cellular proliferation or significant change in DNA synthesis, when compared to control or DFMO treated cells during the first 24 hours, again supporting the association of c-myc with the differentiation state of the cell.

(B) Modulation of Albumin Gene Expression in the Rat Hepatoma Lines Reuber and 7777 by Sodium Butyrate and ADP-ribosylation. Chromatin conformation plays a key role in the regulation of gene expression. The use of chemical compounds which alter the chromatin structure has helped to elucidate the relationship between conformational modifications and gene expression. A strong correlation between transcriptionally active chromatin and acetylated histones has been reported in a number of eukaryotic systems following treatment with sodium butyrate (BA). However, there is equally strong evidence for the inactivation of genes, specifically the hormonally induced gene expression of ovalalbumin and transferrin in oviduct explants and tyrosine aminotransferase in HTC cells following treatment with BA. Thus, although histone hyperacetylation is associated with the regulation of gene expression, its functional specificity and relationship to other controlling elements is presently unclear.

The involvement of poly ADP-ribosylation of histone proteins in the regulation of gene expression has also been studied in several cell systems using two inhibitors of poly ADP-ribosyl transferases (ADPRTs), nicotinamide and 3-aminobenzamide (3-AB). For example, in the 341 mouse carcinoma cell line, the glucocorticoid inducible expression of mouse mammary tumor virus was stimulated when cells were incubated in the presence of 3-AB. In contrast, the induction of two oncofetal proteins, γ-glutamyl transpeptidase and the K-type III isoenzyme of pyruvate kinase, following in vitro culturing of primary adult rat hepatocytes, was inhibited when

cells were incubated in the presence of 3-AB or nicotinamide. Thus, although the modification of histone and nonhistone proteins by poly ADP-ribose appears to effect the expression of certain genes, the synergistic effect of other positive and negative regulators appears to be important.

To further understand the relationship between chemically-induced conformational changes of chromatin and the expression of specific genes, the effects of BA and inhibitors of poly ADPRT on two liver specific genes that are associated with both normal differentiated hepatocytes (i.e., albumin gene) and the oncofetal stage (i.e., α-fetoprotein (AFP) gene) were investigated in the Reuber rat hepatoma line, H4-II-E, and in the 7777 rat hepatoma line. These two cell lines differ in that the albumin gene is expressed in high quantities in the Reuber cell line and low quantities in the 7777 cell line while AFP is expressed in low quantities in the Reuber cell line and high quantities in the 7777 cell line. We have demonstrated that the albumin gene, in both cell lines, was specifically affected by the treatment with BA and inhibitors of ADPRT. In the Reuber cell line, a 55-fold increase in albumin secretion and an approximate 10-fold increase in the level of albumin specific RNA was demonstrated following treatment with 3 mM BA for 72 hours. Unlike the inhibitory effect of BA on the induction of tyrosine aminotransferase activity by dexamethasone in HTC cells, the effect of BA on albumin synthesis in Reuber cells was not rapid (hours vs minutes) showing no change in albumin secretion for up to a 3 hour post-treatment period, when compared to untreated controls, and reaching a maximal role of albumin secretion of 72 hours post-treatment. No affect on the levels of AFP specific RNA was observed in either cell line following treatment with BA.

The effect of inhibitors of ADP-ribosylation was next determined in cells maintained in the presence or absence of BA. Treatment of Reuber cells with 3 mM 3-aminobenzoic acid (3-AB) dramatically reduced the level of albumin specific RNA. Similar decrease was also observed following treatment with 10 mM nicotinamide, another inhibitor of poly ADPRT. Incubation in the presence of the non-inhibitory analogue, meta-aminobenzoic acid at 3 mM did not affect the levels of albumin specific RNA. Interestingly, the inhibitory effect of 3-AB on albumin RNA levels was overcome to some extent when cells were coincubated in the presence of both 3-AB and BA. These results were also reflected in albumin secretion, as measured by radioimmunoassay. BA also appeared to have a direct positive effect on the levels of poly ADP-ribosylation in Reuber cells when assayed using the in vitro nuclear system in the presence of 3 M NAD. No effect on the AFP specific RNA was demonstrated under this treatment protocol.

Thus, we have a system in which a "differentiation" specific gene is regulated by two chemical modulators of chromatin, BA and inhibitors of only ADP-ribosylation. This effect is seen in two cell lines whose base level of albumin specific RNA are very different, suggesting that the effect of BA and inhibitors of poly ADP-ribosylation are acting specifically on this differentiation associated gene rather than on a gene which is normally "turned on." We are presently trying to determine if treatment with BA has any direct effect on the DNAse I sensitivity patterns for the albumin gene vs the AFP genes, thus giving additional information concerning the specific interactions of BA on chromatin.

3. Molecular Cloning of Genes Associated with Chemical Hepatocarcinogenesis

A. Isolation of cDNA Recombinant Clones Which are Up-Regulated in Actively Proliferating Rat Liver. A cDNA library was constructed from poly(A) RNA obtained from

3. Molecular Cloning of Genes Associated with Chemical Hepatocarcinogenesis

A. Isolation of cDNA Recombinant Clones Which are Up-Regulated in Actively Proliferating Rat Liver. A cDNA library was constructed from poly(A) RNA obtained from 18-hour posthepatectomized (70%) Fischer rats. From this cDNA library a clone bank of 6,000 colonies was isolated and screened by the method of differential hybridization to identify clones that corresponded to genes which are specifically "turned on" 18 hours after 70% hepatectomy as compared to "resting" adult liver. No qualitative changes were seen in gene expression; however, four unique clones were isolated, containing cDNA inserts which varied from 600-2,000 base pairs (b.p.) which were up-regulated in regenerating liver. One of the clones has been initially characterized. It consists of approximately 1,080 b.p. and by slot blot analysis to poly(A) RNA has been shown to be three times more abundant in 18-hour posthepatectomized liver than sham-operated controls. In addition, Northern blot analysis has shown that this clone is also up-regulated in the livers of rats chronically fed phenobarbital or injected with 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), two potent tumor promoter regimens. In contrast, this gene was down-regulated in frank liver tumors, preneoplastic nodules produced by the Solt-Farber method, in the rat hepatoma line 7777 and liver which has completely regenerated. Although this gene has not yet been identified, certain possibilities have been eliminated. Southern blot analysis of the cDNA insert has shown no homology to rat P-450 enzymes, inducible by 3-methylcholanthrene or phenobarbital, ornithine decarboxylase, or PRO-2, a promoter related gene in the epidermis. We are presently recloning this gene in M13 for nucleic acid sequence analysis and will further characterize this gene following isolation in a recently constructed rat genomic library.

B. Construction of cDNA libraries in λ-gt II. The cDNA library of choice which we are presently utilizing is the λ-gt II system. Briefly, this system offers several long range advantages. The cDNA is linked to two arms of the λ gt II phage and packaged as a recombinant phage. The phage is infected onto appropriate indicator bacteria and grown in the presence of the chromogenic substrate X gal. Wild type phage are identified by their ability to utilize X gal following derepression of their lac operon (IPTG) and will be blue. Recombinant phage which has interrupted their lac operon by insertion of the cDNA will be unable to utilize X gal and will be identified as clear plaques. The efficiency of this system in our hands is 1×10^7 recombinants/μg cDNA. We presently have two libraries to (a) normal rat liver and (b) Solt-Farber induced rat hepatoma containing between 50,000-75,000 recombinants. In addition to high yields, the bacteria will also make a fusion protein, containing at least part of the protein coded by the inserted cDNA, thus allowing identification of clones by monoclonal antibodies. We hope to apply this technique to antibodies raised against unique proteins identified and isolated using the two-dimensional gel system. In addition, other members of the laboratory are purifying and sequencing proteins of interest from which synthetic DNA probes can be made and used to identify clones in these libraries. Finally, a genomic library was constructed to the Fischer rat genome using the EMBL vector system which can also be used for further analysis of the genes of interest.

C. Cloning of Rat γ-Glutamyl Transpeptidase (GGT) Gene. The heavy and light subunits of rat kidney GGT were purified to homogeneity after papain treatment. N-Terminal amino acid sequencing of both fragments was performed and the first 32 and 29 amino acids from the heavy and light subunits, respectively, were identified. Six amino acid sections of both the heavy and light GGT subunits

were chosen for synthesis of the corresponding oligonucleotide series (17-mers). Due to the total amount of "wobble" in the light subunit, two different oligomers were synthesized, differing only at position 9.

A rat genomic library made in Charon 4A (a gift of Dr. T. Sargent, Laboratory of Molecular Genetics, NICHD, was plated on NZCYM-agar plates using bacterial strain LE392 as host (16,000-20,000 plaques/plate). Plaques were transferred to nitrocellulose in triplicate, denatured in 0.5 M NaOH, 1.5 M NaCl and neutralized in 0.5 M Tris HCl, pH 7.5, 1.5 M NaCl. Nitrocellulose filters containing DNA were baked at 80° in vacuo for 2 hours. Synthetic oligonucleotides were end-labeled using ^{32}P-γ-ATP and T4-polynucleotide kinase and used to screen the library for GGT.

Out of 480,000 plaques screened, 1 plaque was positive for both the heavy and light subunit probes. The phage from the positive plaque (#64) were replated on NZCYM-agar plates (at a low density), transferred in triplicate to nitrocellulose filters and rescreened with the heavy and light subunit probes, as described above. On the secondary round of screening, all plaques derived from the original positive plaque #64 were again positive for probes made from both subunits.

Large cultures of phage from plaque #64 were grown and the phage purified using CsCl gradients. The DNA was then extracted and used for further characterization of this clone.

GGT-DNA was cleaved using a series of restriction enzymes, run on agarose gels, and stained with ethidium bromide. EcoR1 cleavage of the DNA revealed four bands, two representing the arms of vector DNA and two representing the insert containing an internal EcoR1 site. The two bands of the insert migrated with molecular weights of approximately 5.8 and 7.2 kb. Cleavage with other enzymes gave a series of bands of varying sizes. DNA fragments were then transferred from the gels to nitrocellulose and baked for two hours at 80° in vacuo. Screening of the restricted DNAs revealed that an EcoR1 fragment (~ 7.2 kb), an EcoRV fragment (~ 2 kb), and a Hind III fragment (~ 1.2 kb) hybridized to the heavy and light subunit probes. The smaller EcoRV and Hind III fragments are being subcloned into pBR322 for further mapping, sequencing and gene expression studies.

4. Cellular Evolution of Chemically Induced Murine Hepatomas

(1) Transplantation of hepatocytes into the anterior chamber of the eye allows the cells to survive for extended periods that are needed for studying the different stages of hepatocarcinogenesis. (a) Normal hepatocytes attach and proliferate in the anterior chamber. The proliferation is enhanced by the humoral effect of partial hepatectomy. (b) Tumor promoters are toxic for the normal hepatocytes growing in the anterior chamber. However, the growth of preneoplastic and neoplastic hepatocytes is enhanced by tumor promoters. (c) Preneoplastic liver cells may be concentrated by their failure to adhere to tissue culture plates coated with asialofetuin; such cells show a definite growth advantage in the anterior chamber of the rat eye when promoted by phenobarbital. (d) Hepatocytes isolated from animals treated with ultimate carcinogens intraportally a few minutes before the perfusion of the liver and transplanted to the anterior chamber of the eye survived for several months. However, the thymidine labeling index and the histological staining for gamma-glutamyl transpeptidase did not reveal signs of malignant transformation. In vitro treatment of hepatocytes with ultimate carcinogens gave similar results.

(2) The effect of the liver specific tumor promoter, phenobarbital on asialoglycoprotein receptor (ASGP-R) was examined in adult rat liver. Both acute and chronic phenobarbital administration decreased the number of receptors per cell determined by radioreceptor assay. Immunofluoresence histochemistry of liver samples from phenobarbital treated animals revealed centrilobular receptor-deficient areas. In contrast, after partial hepatectomy ASGP-R positive and negative areas were intermingled throughout the liver lobule. Preneoplastic and neoplastic areas displayed uniform reduction in ASGP-R. Four days after birth, the number of hepatocytes with surface receptors was 50% of that in the adult rats. At 10 days after the birth the number of ASGP-R positive cells was the same as in adult rats, although the receptor density was significantly lower than in adults.

(3) Cell differentiation is defined on the basis of cell morphology. In situ hybridization takes into account the morphological aspects of cell differentiation and the changes in the gene expression in these cells. Cytohybridization is a histochemical procedure which combines traditional histology with methods used in molecular biology. It has been used successfully to localize virus derived RNA and DNA molecules. In the earlier methods signal detection was limited to abundant mRNAs that constitute 1 to 5% of the total poly(A)+RNA population. The development of nick-translated high specific-activity c-DNA has increased the sensitivity of in situ hybridization to include 0.1-0.01 % of the total poly(A)+RNA. Thus a high resolution mapping of intracellular mRNA is now possible. However, depending on the tissue of interest, the conditions for in situ hybridization must be optimized to obtain high signal to noise ratio. Our present goal is to determine (a) appropriate fixation methods for liver samples, (b) appropriate coating methods for slides and cover-slips to obtain sufficient attachment of frozen tissue sections and to prevent non-specific binding of radioactive probes, (c) appropriate proteolytic digestion of frozen sections to allow penetration of the cell membranes by the probes and still maintain the normal morphology of the section, (d) optimal conditions for hybridization (temp. time, etc.), and (d) optimal conditions for washing of slides to remove nonspecifically bound radioactive probes. The ultimate goal of the technique is to retain the labile mRNA in the liver cells and overcome the hindrance of accessibility of radiolabeled probe for cellular mRNA and simultaneously preserve the intact anatomical structure of liver for subsequent identification of transformed liver cell areas. Our principal goal is to use in situ hybridization to gain insight into the factors that influence the level of specific mRNA in hepatocytes of intact animals and under the influence of carcinogens and liver tumor promoters. This will be combined with immunohistochemical localization of gene products in the same cells using specific antibodies.

5. Hepatocellular Carcinoma: Expression of Retroviral Associated Oncogenes

A. The Tumorigenic Characterization and Oncogene Expression in a Human Hepatoma Cell Line, HEP G2. HEP G2 is a human cell line derived from a primary hepatocellular carcinoma. This cell line retains some biosynthetic capabilities of normal liver parenchymal cells as well as the capacity to metabolically activate a variety of chemicals, such as cyclophosphamide. We have demonstrated that this cell line is tumorigenic when injected subcutaneously into athymic nude mice, with the tumorigenicity being dependent on the number of injected cells. Tumors were nonencapsulated, highly invasive adenocarcinomas and were positive for gamma-glutamyl transpeptidase activity and bile production. Plasma from tumor bearing mice were positive for human alpha-fetoprotein indicating the human

origin of the tumors, but serum was negative for hepatitis B virus surface antigen as measured by radioimmunoassay. Cyclophosphamide (CY) pretreatment of HEP G2 cells (500 μg CY/ml/two cell cycles) significantly elevated the number of sister chromatid exchanges indicating significant DNA damage, but had no effect on tumor incidence or latency when these CY pretreated cells were injected into nude mice. Two cell lines were reestablished into tissue culture from HEP G2 derived tumors and were shown to have similar cellular morphology and unaltered cell cycle times when compared to the parent HEP G2 cell line.

This HEP G2 system, using normal human liver as a control, provides a model for identifying the genes and gene products that are associated with the tumorigenic phenotype. To demonstrate that these cells still maintain an overall genetic similarity to normal human liver, poly(A)RNA isolated from HEP G2 derived cells and tumors were in vitro translated using a cell-free rabbit reticulocyte lysate system. HEP G2 translation products, when analyzed by two-dimensional polyacrylamide gel electrophoresis, were extremely similar to the translation products from poly(A)RNA isolated from a normal human liver sample, except for one 53,000 molecular weight polypeptide with an apparent charge shift.

As a first step in identifying the genes which bestow the transformed phenotype to the HEP G2 cells, expression of proto-oncogenes (cellular oncogenes) implicated in other human malignancies was examined. C-myc specific transcripts, when compared to a normal human liver sample, were increased in all HEP G2 cell lines and tumors derived from HEP G2 cells. Contrary to other systems, the elevation in HEP G2 c-myc transcripts could not be superinduced by cycloheximide treatment. Southern blot analysis indicated that the increase in c-myc specific transcripts could not be explained by myc gene amplification or hepatitis B virus integration into the HEP G2 genome. These data suggest that transcription of the human c-myc gene is elevated in both cultured cells and transplantable tumors of HEP G2 origin. However, other possibilities such as specific stabilization of c-myc mRNA cannot be excluded.

Since DNA transfection experiments in rat embryo fibroblasts suggest that two genes from two distinct complementation groups of oncogenes or viral elements are required for cell transformation, we examined HEP G2 derived cells and tumors for the expression of an oncogene family, ras, which is complementary to the myc gene for cell transformation. C-Ha-ras expression could not be detected in any HEP G2 derived tumor or cell lines. Equal levels of 5.5 kb and 2.5 kb N-ras specific transcripts were detected in all HEP G2 derived cell lines and normal liver samples. However, RNA isolated directly from the tumors produced by HEP G2 cells had approximately a five-fold increase in the 5.5 kb N-ras specific transcript. This selective increase in the 5.5 kb N-ras transcript was reproducibly elevated in three tumors that were tested. Since this increased 5.5 kb transcript did not remain elevated if the tumors were reestablished into tissue culture, it suggested some interaction with the host animal, such as with a serum growth factor or hormone.

It is important to note, however, that an increase in N-ras specific transcripts may not be critical to complement the increased c-myc expression in tumor formation if the ras gene has been activated by a point mutation. Recent experiments have shown that the N-ras gene of HEP G2 cells is capable of transforming NIH/3T3 cells in a DNA transfection assay (Notario et al., Cancer Cells, p. 425. Cold Spring Harbor Laboratory, 1984). This indicates that the gene is activated, since unmutated N-ras genes derived from normal human cells are not capable of

transforming NIH/3T3 cells in this system. Hence, normal transcriptional levels of the activated N-ras gene may complement the increased c-myc transcripts in the tumorigenic properties of HEP G2 cells. This hypothesis is based on the assumption that the HEP G2 N-ras gene is translationally active and produces a gene product, P21, which has different properties from an unmutated N-ras gene product (i.e., GTPase activity). Immunoprecipitation of S-35 labeled HEP G2 cell lysates with anti P21 antibodies demonstrated a specific immunoprecipitable P21 polypeptide.

B. Gene Expression during Development of Chemically Induced Hepatocellular Carcinoma in the Rat. An important feature in the development of many if not all human and experimental tumors, including hepatocellular carcinoma, is the appearance of preneoplastic lesions which may undergo additional alterations during the progression to cancer. For this reason we have utilized an *in vivo* rat model to study gene expression sequentially throughout chemically induced liver carcinogenesis. The rat liver has been used extensively as an experimental model in carcinogenesis studies and allows identification of preneoplastic cell populations very early in the neoplastic process. To induce preneoplastic and neoplastic lesions the method of Solt-Farber was used, which includes i.p. administration of diethylnitrosamine (200 mg/kg body weight) for initiation and partial hepatectomy combined with low dose feeding of acetylaminofluorene (0.02%). An early preneoplastic alteration in hepatocytes is the appearance of focal cell populations with a greatly reduced concentration of the asialoglycoprotein receptor, a hepatic cell surface receptor which binds and internalizes desialylated serum glycoproteins, such as asialofetuin. Preneoplastic focal hepatocyte populations were isolated from collagenase perfused liver cell suspensions by their inabilty to bind to tissue culture plates coated with asialofetuin.

Gene expression was compared and contrasted in normal rat liver, preneoplastic foci, preneoplastic nodules, and primary hepatocellular carcinomas by Northern blot analysis. Initially, the expression of four genes was examined in detail: ornithine decarboxylase (associated with proliferation); alpha-fetoprotein (associated with a less differentiated state); P-53 (associated with the transformed phenotype); and myc (an oncogene associated with certain human maligancies including hepatocellular carcinoma [see above]). Compared to normal rat liver, no change in specific transcripts of any of the four genes was found in the preneoplastic foci. Preneoplastic nodules had slightly elevated ODC transcripts but all other genes remained unchanged. Neoplastic tumors had greatly elevated ODC transcripts (to the levels observed 24 hour post 70% hepatectomy), elevated P53 transcripts, just detectable AFP transcripts, and detectable transcripts which hybridize to a v-myc probe only under moderately stringent conditions. These tumors were characterized by a great deal of heterogeneity with regards to expression of the four genes analyzed.

C. Transcriptional and Post-Transcriptional Regulation of the Asialoglycoprotein Receptor (ASGPR). The ASGPR is a hepatocyte cell surface receptor which binds and internalizes serum glycoproteins with galactose-terminal carbohydrate chains. Recent experiments have demonstrated that cell-surface binding activity and cell-surface receptor proteins are decreased or absent in fetal, regenerating, phenobarbital treated and neoplastic liver, while binding activity is increased in livers of pregnant dams. We have capitalized on this phenomena to isolate preneoplastic foci in carcinogen treated rat livers (see B above). To understand more about this phenomena, we investigated the genetic regulation of the ASGPR by isolating poly(A)RNA from fetal, neonatal, regenerating, phenobarbital treated

and chemically induced neoplastic Fischer rat livers. When compared to adult liver, the amount of ASGPR specific transcripts showed no change at 1, 3, 12, 24 and 64 hour post 70% hepatectomy; in fetal, neonatal and livers of pregnant dams; in livers of phenobarbital treated rats; and in chemically induced (Solt-Farber) preneoplastic and neoplastic liver samples. These data suggest that, in vivo, the ASGPR is regulated post-transcriptionally, possibly at the cell membrane. Interestingly, no transcripts could be detected in a Morris hepatoma cell line, 7777, despite being able to detect the ASGPR gene by Southern blot analysis. This indicates that in tissue culture, the ASGPR gene could be regulated at the transcriptional level. In addition, preliminary data indicates that freshly isolated hepatocytes, when placed into tissue culture, have significantly lower ASGPR specific transcripts within approximately 2 hours.

6. Cell Surface Proteins and Cellular Adhesion in Hepatocarcinogenesis

The main objective of this project involves analysis of changes in homotypic cell to cell adhesion during different phases of chemically induced rat hepatocarcinogenesis. The results so far obtained include: (1) Hepatic cells obtained by perfusing livers with collagenase at different stages of chemically induced (Solt-Farber and/or Peraino method) hepatocarcinogenesis exhibit differential adhesive properties: cells obtained at the preneoplastic nodule stage are more adhesive and cells obtained from a neoplastic stage are less adhesive than normal liver cells. (2) Transforming growth factor beta (TGFβ), when added to the growth medium of normal rat kidney (NRK) cells, elicits a reduction in the ability of the cells to adhere to each other. Epidermal growth factor (EGF) has the apparent capability of reversing the adhesion-impairing effect of TGFβ. (3) Clones derived from a Fischer rat liver derived cell line show markedly varying intercellular adhesive characteristics. The differences in adhesiveness roughly correlates with the chromosome number in the cells in each clone, so that cells that have acquired an aneuploid modal chromosome number tend to be more adhesive to each other than cells that have remained diploid during the cloning process. 2-D analysis of concavalin A binding proteins in the cells showed that the cells from the "most adhesive clone" had quantitatively more protein than the "least adhesive" clone; qualitative changes were little. Neither the clones nor the parent cell line, up to passage 34, were able to grow on soft agar in the presence or absence of EGF.

7. Metabolism and Mutagenicity of Chemical Carcinogens

Cytochrome P-450 Dependent Metabolism. 1. Studies with Human Tissues. Two distinct phenotypes, slow and fast metabolizers, were observed for both metabolic activation and detoxification of the model chemical carcinogen, 2-acetylaminofluorene, in human liver microsomes from 28 individuals. We observed that individuals who were fast activators of the carcinogen were in most cases also fast detoxifiers of the chemical. However, different phenotype patterns exist suggesting that fast activators of a toxin need not also be phenotyped as fast detoxifiers. Studies in this area may help understand whether certain individuals are predisposed to a higher rate of chemically-induced cancer.

2. Studies with Animal Tissues. (a) The kinetics of cytochrome P-450 dependent N-hydroxylation of 2-aminofluorene (AF) and 4-aminobiphenyl (4-AB) were determined using six highly purified forms of rabbit cytochrome P-450, and microsomes from control and 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) induced rabbit liver and lung. N-Hydroxylation of both AF and 4-AB was best defined by two enzymes systems,

showing a high affinity low capacity and a low affinity high capacity, in control and TCDD microsomes. Pretreatment with TCDD modified the apparent Km and Vmax for the N-hydroxylation of both substrates in liver and lung microsomes, but the biphasic kinetics were observed in all instances. Form 4 was the only form capable of catalyzing the N-hydroxylation of AF and 4-AB. The kinetic data obtained with form 4 for these two substrates were consistent with a single enzyme system.

α-Naphthoflavone (ANF) completely inhibited the N-hydroxylation of AF in liver microsomes from both control and TCDD treated animals, whereas only partial inhibition was obtained with lung microsomes. These results indicate that at least two forms of cytochrome P-450 with greatly different substrate affinity (Km) participate in the N-hydroxylation of primary aromatic amines in rabbit liver and lung microsomes.

(b) The metabolism of 2-acetylaminofluorene (AAF) has been used to study cytochrome P-450 monooxygenase activity in two rat hepatoma cell lines, McA-RH7777 and Reuber H4-II-E. McA-RH7777 cells exhibited considerably higher basal activities than H4-II-E cells for all metabolic pathways studied. Differences varied from 1.8-fold for the 9 hydroxylation to 11-fold for the 3- and 5-hydroxylations of AAF. Both phenobarbital and TCDD caused a marked induction of AAF metabolite formation in both cell lines. However, because of the low basal activities of AAF metabolite formation in H4-II-E cells, the fold induction caused by phenobarbital in these cells still results in activities below those of control McA-RH7777 cells. The half-life of AAF disappearance in control, phenobarbital and TCDD pretreated McA-RH7777 cells were 240, 34 and 23 min, respectively. This is the first time the cytochrome P-450 monooxygenase system of an established cell line has been shown to respond equally to both phenobarbital and TCDD induction.

8. Chemical Transformation of Human Lymphoblastoid Cell Lines

The objective of this project is to characterize the nature of chemically induced transition from a benign hyperproliferative to a malignant state in Epstein-Barr virus (EBV) immortalized human lymphocyte.

1) In Vitro Transformation and Transplantation. Early passage CB and Eckert cells were exposed to the chemicals for 24 hours, washed, replated and viability determined. Viabilities ranged from 86% to 24% with the Eckert line showing the greatest sensitivity to N-acetoxy-2-acetylaminofluorene N-OAc-AAF. After 20 population doublings, cells were injected subcutaneously into 7 to 12 gram athymic mice. Within two weeks palpable tumors (7 mm^3) were found in mice injected with CB23 cells treated with 15 μg/ml of N-OAc-AAF. By three weeks, all high dose (15 μg/ml) N-OAc-AAF treated cultures, as well as 5 μg/ml treated Eckert cell line, caused similar tumor growth in vivo. One untreated CB23 innoculum gave rise to a palpable tumor within two weeks, but had thoroughly regressed by four weeks. Contrary to this, the treated lines gave rise to aggressively growing tumors, reaching sizes of 15 mm^3 in four weeks. One DMSO treated CB34 control gave rise to a histologically identical tumor when compared to those which developed in the N-OAc-AAF treated lines. The MNNG treated lines did not give rise to any cells capable of tumor formation in vivo.

At four weeks growth, the tumors were aseptically excised from the animals. Half of the tumor was taken for histology and half was recultured. After 5 population doublings, the cultured primary tumor lines were reinjected into athymic mice.

Tumor formation occurred within one week this time and the same tumor type was generated. The N-OAc-AAF induced tumors were histologically classified as high grade "immunoblastic" lymphomas showing thickened nulear membrane, round oval nuclei and densely packed cells with prominent nucleoli.

2) Cell Surface Characteristics and Karyotypes of the Cell Lines of N-OAc-AAF Induced Tumors. The cell cultures derived from the tumors were assessed for cell surface characteristics by fluorescent activated cell sorting analysis. The cells were all of the B lineage as determined by the presence of surface immunoglobulins and antigens detected by antibodies to B_1 and B_4, and the absence of $3A_1$ and LEU-1 reactivity in those cultures tested (T-cell associated). Apparently, clonal selection of the cells has occurred in vivo as noted by the change in predominant light chain production in the untreated parental lines, CB23 and Eckert and the treated tumor counterparts. The simultaneous presence of B_1, B_4 and surface immunoglobulins suggests that these cells are of an intermediate maturity, between pre-B cells and plasma cells. Three of the tumor lines (A23-15, A34-15, and D34) have begun to produce surface IgD, suggesting that immunoglobulin maturation and DNA rearrangements may be occurring.

Karyotyping of the CB and Eckert lines, after 30 population doublings in vitro, revealed an essentially normal diploid to slight aneuploid chromosomal complement, with CB23 displaying a modal chromosome number of 47 (80% of all cells). On the other hand the N-OAc-AAF induced tumor lines displayed an abnormal diploid to tetraploid karyotype. The abnormal diploid karyotype found in one of the N-OAc-AAF induced tumors ($A23\text{-}15_1$) was characterized by deletion in chromosome 6, and additions in chromosomes 16 and 4. The other two N-OAc-AAF induced tumors, as well as the spontaneously occurring tumor, showed tetraploid karyotypes.

3) Oncogene Expression of Rearrangement in N-OAc-AAF Induced Tumors. Since the karyotype revealed N-OAc-AAF induced chromosomal rearrangements, the possibility that oncogene rearrangement might have occurred was investigated. Southern blot analysis of total restricted genomic DNA derived from both the untreated parental lines and the N-OAc-AAF induced tumor lines was assessed for major rearrangements in oncogenes associated with human lymphomas (c-myc and Blym) or with fragile sites on chromosome 6, c-myb. Similar analysis was also performed for the ras family members, K-ras, H-ras, and N-ras, since these genes are commonly associated with human tumors, and one of these genes (i.e., c-K-ras-1) has been localized on human chromosome 6 (6p11-12, N. Popescu, personal communication). Under these conditions, no differences in arrangements were noted, nor was amplification of any of these oncogenes found as illustrated by the Southern blot analysis to N-ras. Total poly(A)+ RNA was isolated from both sets of cell lines and analyzed by Northern blots. Again no increase in expression of any of the above oncogenes was observed.

EBV copy number was also determined by slot blot analysis and contrary to other reports concerning chemical carcinogen treatment of EBV immortalized CB lines, no increase in EBV viral DNA was observed. Conversely, a slight decrease in copy number, of approximately 10 copies per genome, was noted in all the chemically transformed lines when compared to their nontransformed counterparts.

4) Transforming Genetic Element(s) in N-OAc-AAF Induced Lymphoma. In order to determine the transforming genetic element(s) in our chemically induced lymphoma cells, we have transfected high molecular weight DNA derived from both the

N-OAc-AAF treated CB23 (A23-15), and the original CB23 cell lines into NIH3T3 and have screened the resulting foci for the presence of oncogenes, including EBV fragments.

The first round of transfection with DNA isolated from A23-15 cells gave rise to typical multilayered, well-defined foci. No foci were seen following transfection with DNA from the original untreated CB23 cells. Southern blot analysis of genomic DNAs derived from these transfected foci revealed the presence of human DNA as noted by Alu specific repeat sequences, yet none of the above mentioned oncogenes were found in any of the transfected cells.

The Cell Biology Section plans and develops laboratory research aimed at determining the cellular events which mediate the conversion of normal cells into neoplastic or cancer cells. The major efforts of the Section are presently focused on (1) application of the transgenic mice system to introduce specific gene(s) into the germline of the murine genome and to study the expression, the interaction with chemical carcinogens, and the biological consequences of the introduced gene(s) in the host animal throughout development; (2) development of in situ hybridization and immunochemical staining methods to study tissue specific and developmental specific gene expression; (3) application of the retroviral vector system to introduce genes in liver and germ cell lineage in vitro and in vivo and to study the role of introduced gene(s) in oncogenesis; and (4) conduct parallel studies for the identification of genes acting both in normal development and in the neoplastic process of hepatoma and teratomas using advanced recombinant DNA and monoclonal antibody techniques.

1. Normal Stem Cell Biology and Hemopoietic Regulation

We have used our previously identified erythroleukemia cell lines as a source of the hormone for the purpose of purifying erythropoietin to apparent homogeneity. A relatively rapid and simple purification scheme was developed and involved lectin chromatography, gel filtration, ion exchange chromatography and hydroxylapatite chromatography, and finally high pressure liquid chromatography. Polyarylamide analysis indicates that this material is pure.

2. Analysis of Direct Actions of Transforming Proteins of Oncogenic Viruses

We have continued our study of the effects of virus transforming genes on hemopoietic cells. We have now been able to prepare retroviral vectors which carry genes into specific types of hemopoietic cells. This will allow the study of many of the questions associated with tissue specific gene regulation, enhancer elements, etc. In particular, we now can determine the effects of putative regulatory sequences in erythroid precursors as they differentiate. This is possible because of our demonstration that vectors carrying p21 genes become integrated into and are expressed in erythroid precursors after direct infection in vitro.

We have now moved on to ask whether it is possible to infect a single multipotential cell and if so, whether the genes are expressed in the different lineages which are the progeny of that cell. A manuscript is in preparation detailing this work which clearly demonstrates that the viral gene ras can be transferred into a single hemopoietic stem cell. When the cell is subcultured under the appropriate hormonal conditions to allow blood cell differentiation, p21 expression in some, but not all, progeny is unequivocal.

3. Gene Expression and Development in Transgenic Mice

The overall objective of this project is to exploit the transgenic mouse system by introducing natural or manipulated gene sequences into the germline of an animal and to alter its phenotype and genetic background. This system provides a new way of investigating tissue specific and developmental stage specific regulation of gene expression. The current research is focused on three classes of genes that may be initially associated with the multistage process of murine liver tumorigenesis: (1) cytochrome P-450 genes involved in metabolic activation of chemical carcinogens; (2) the known oncogenes, myc, ras and SV40 large T antigen, and (3) the developmental expression of a family of genes that contain sequences homologous to the Drosophilia homeobox. At present the work has involved setting up the transgenic mouse system.

The Biopolymer Chemistry Section plans and develops laboratory research on the chemical structure and conformational aspects of relevant biopolymers, of reactant chemical carcinogens and of carcinogen-biopolymer adducts using rigorous spectral and chemical methods such as mass spectrometry, nuclear magnetic resonance spectroscopy, circular dichroism, chemical synthesis and related techniques. The major efforts of the Section focus on (1) the isolation, characterization and mass spectral sequencing of polypeptides (proteins) that are determinants in the control of cell differentiation and proliferation; (2) the mechanism of action of these biopolymers; and (3) the synthesis and reactivity of carcinogens with biomolecules, particularly with DNA, and the structure, conformation and biological significance of the resulting modified products. Studies of the Section are aimed at a better understanding of the carcinogenic process at the molecular level in particular.

1. Effect of Chemical Leukemogens on Hemopoietic Target Cells

(1) We have initiated the in vivo experiments in which female Long-Evans rats were given four intravenous pulse doses of DMBA and TMBA at biweekly intervals beginning at age 28 days. Hematologic examination is routinely performed on blood drawn by the tail vein under brief ether anesthesia to determine the onset of leukemia. Mammary carcinoma have recently been detected in a number of TMBA treated rats.

(2) We have successfully developed primary cell culture clonal assays detecting both rat and mouse hemopoietic progenitor cells which form pure and mixed colonies consisting of mature and morphologically identifiable cells of more than four lineages, as well as blast cell colonies which are composed of only undifferentiated blast cells. In the secondary cell culture clonal assays, these blast cells can give rise to pure and mixed colonies, many of which consist of cells of three to four lineages.

(3) We have used these assays to study the interactions of chemicals on the proliferation and differentiation of hemopoietic cells in vitro. Treatment of marrow and spleen cultures with chemical leukemogens including DMBA, MNU, N-OH-AAP and the hepatocarcinogen, N-OAc-AAF showed no effect on the colony formation of an array of normal murine hemopoietic progenitor cells, with the exception of non-specific cytotoxicity at high concentrations. The tumor promoter TPA, however, stimulates the proliferation and differentiation of normal unipotent (erythroid; macrophage) and bipotent (erythroid/megakaryocyte; granulocyte/macrophage) hemopoietic progenitors but has no effect on the multipotential progenitors and

the undifferentiated blast cells. The modulating effect of TPA on the late progenitor cells appears to be mediated via altering the responsiveness of progenitor cells to growth regulators or by inducing accessory cells to secrete growth factors rather than acting directly on the progenitor cells.

(4) We are currently investigating the in vivo effect of DMBA and TMBA on murine hemopoietic target cells by determining the colony formation ability in cell culture of hemopoietic progenitor cells isolated from treated animals.

(5) Administration of [ring-3H]-DMBA to Long-Evans rats in single intravenous doses resulted in substantial binding of DMBA to DNA isolated from hemopoietic tissues including bone marrow and spleen. DMBA-DNA adducts were found to persist in the spleen but rapidly lost from the bone marrow. Structural confirmation of individual DMBA-DNA adducts are presently characterized.

2. Structural and Physicochemical Studies of Proteins Relevant to Tumorigenesis

(1) Chemical structure studies on gamma-glutamyl transpeptidase (GGT). gamma-Glutamyl transpeptidase (GGT) is a membrane bound glycoprotein enzyme of unknown structure that is involved in the degradative metabolism of glutathione and the uptake of some amino acids. In rodents GGT activity is especially high in fetal liver and in adult kidney but not in adult liver. The low liver enzyme activity is inducible by certain drugs and carcinogens such as azo dyes. For example, elevated levels of GGT are found in most hepatomas. This enzyme is thus expected to be a marker of neoplastic transformation, especially in hepatoma cells. It is also known that there are structural differences between the enzymes found in tumorigenic tissue and the corresponding normal tissues.

All structural work was carried out on an enzyme isolated from normal adult rat kidneys, so that the data can serve as a basis for comparison of the enzyme from other sources. A 170-fold enzyme purification was achieved providing an electrophoretically pure preparation. It is known from earlier work that the enzyme is a heterodimer. Our selected method of purification involved freeing the enzyme from the short membrane binding hydrophobic segment of the heavy subunit with papain, followed by various chromatographies. A fast semimicro preparative HPLC method was developed to separate the two enzymatic subunits under acidic conditions, but in the absence of detergents, buffers and denaturants, thus providing suitable samples for chemical sequencing. 2-D gel electrophoresis was found to resolve the active papain purified enzyme into at least 18 components. Seven components with apparent molecular weight of 23-26,000 and isoelectric point range of 5.4-7.0 comprise the light subunit, and 11 components with apparent molecular weight of 51-53,000 and isoelectric point range of 5.8-7.1 comprise the heavy subunit. Immunoblot analysis of 2-D gels showed that all of these components are immunoreactive with a mixture of the two antibodies generated separately against the light and heavy subunits, demonstrating that they are all valid constituents of the enzyme complex. N-Terminal amino acid sequencing of the separated subunits of the papain purified enzyme yielded for the first time sequence information for the first 32 residues of the heavy chain with terminal starting sequence of Gly-Lys-Pro-Asp-His-Val-Tyr-Ser-Arg-Ala and for the first 36 residues of the light subunit with terminal starting sequence of Thr-Ala-His-Leu-Ser-Val-Val-Ser-Glu-Asp. The noncovalently bound light subunit is surprisingly nonpolar, 19 of its amino acids being hydrophobic, and only 5 being the charged type. Whereas the 2-D gel electrophoresis results indicate considerable heretogeneity for the active enzyme, the sequencing information, at least to the extent

determined here, confirms earlier observations that the heterogeneity does not reside in the amino acid sequence of the enzyme, but rather in the attached carbohydrate chains.

(2) Peptide sequencing by fast atom bombardment mass spectrometry. The classical Edman sequencing of proteins and peptides is a time proven technique for structural analysis. There are numerous situations, however, where this technique fails or where the results are ambiguous. In those cases the rapidly developing technique of fast atom bombardment (FAB) mass spectrometry can serve as an invaluable alternative. FAB mass spectrometric measurements allow determination of molecular weights to within 1 mass unit or better on peptides with amino acid residues of 25 and possibly more, a molecular weight range eminently suited, for example, for tryptic peptide analysis. For example, we successfully observed the molecular ion for the 34 residue synthetic fragment of the p21 ras protein (prepared by C.-H. Niu of our laboratory). It would be desirable to extract sequence information also from the mass spectra. Unfortunately, this information, especially when obtained on subnanomole amounts of sample, is not satisfactory. In an effort to overcome this limitation we are developing methods with model peptides, using group specific reagents such as those described below, to increase the predictable charge localization in the molecule once it is ionized, and also to simplify the fragmentation pattern in an effort to give more reliable sequence information.

One set of important N-terminally blocked peptides that poses problems with the classical Beckman method of sequenation is the N-terminal pyroglutamates. We have examined the spectra of several synthetic pyroglutamates in the positive FAB mode of operation. Data indicate that the ionization efficiency of these compounds is lower than it is for underivatized peptides. There is, however, valuable sequence information in the spectra. For example, in the spectra of the nonapeptide, serum thymic factor, charge retention appears on the amino terminal end of the molecule showing informative serial fragments with cleavage of the amide nitrogen and the α-carbon atom of the neighboring amino acid. We are also planning to apply the bromobenzoylation procedure, which we have used more extensively in the past and reported on in last year's reports, for tagging basic sites in these peptides, and thus to be able to locate the sequence position of such sites. Bromine containing tags can also be attached to the carboxy terminal end of peptides by making, for example, the p-bromophenacyl ester derivatives. The bromine atom in these derivatives can act as a marker, since it consists of an even mixture of two isotopes two mass units apart, and thus ions containing the amino terminal segment of the peptide will show up as doublets. Examination of the spectra of a number of underivatized peptides indicates that the low molecular weight region of the spectrum may contain valuable information about the actual amino acid content of a peptide. The principal ion in this connection is an iminium ion of the type $RCHNH_2^+$. Among others phenylalanine, leucine, valine and tyrosine give especially significant ions. The methodology developed in our laboratory is directly applicable to relevant structural problems. We have applied it to date for the structural confirmation of synthetic peptides, prepared in our laboratory, and it should prove to be useful in complementing the classical Edman methodology and the dipeptidase method of GC-MS sequencing approaches.

3. Purification of Rat Hepatic Proliferation Inhibitor (HPI)

The first goal in the effort to purify HPI was to develop a rapid and simple assay that would allow the screening of many fractions at once. With this objective accomplished, a major effort was then focused on defining a route for purification of sufficient amounts of HPI so that the studies described under Objectives could be carried out. This second goal now appears to have been met. Starting with a previous preparative scheme for HPI, a totally new procedure has been developed for isolation of this molecule. The purification now used employs the following steps: 1) homogenization of livers in a Waring blender followed by a second homogenization with a high speed Polytron type of instrument; 2) acidification of the homogenate, centrifugation, neutralization of the supernatant, and centrifugation; 3) ammonium sulfate precipitation followed by ethanol precipitation; 4) phenyl sepharose, then gel filtration column chromatography; 5) FPLC cation exchange chromatography followed by FPLC anion exchange chromatography; and 6) weak anion exchange HPLC. One dimensional gel electrophoresis after anion exchange FPLC showed one major band and several very minor bands in addition to a major band at the position where HPI would be, and after weak anion exchange HPLC showed essentially only one band corresponding to the position of HPI.

Some studies were carried out with partially purified HPI. Stability experiments showed HPI to be rather acid stable, with half the activity retained after 2 hours at pH 1.5 at room temperature, and the same result was obtained after treatment with dithiothreotol. HPI was found to be stable after treatment at 50°C for 2 hours but all activity was abolished at 70°C for 2 hours. This molecule was also rather unstable toward organic solvents at low pH, which precluded purification in an active form by reverse phase HPLC. Attempted chromatography on a variety of polysaccharide binding columns indicates HPI probably has no post-translational modification with oligiosaccharide side chains. The results obtained on the one dimensional gel analyses, which were carried out under reducing conditions, shows that HPI probably consists of a single polypeptide chain of about 25,000 daltons in size. All of these results and several anti-TGF beta antibody studies showed that the HPI that has been studied here is a different molecule than TGF beta, another inhibitor of hepatocyte proliferation.

4. Isolation and Characterization of Proteins from Two-Dimensional Polyacrylamide Gels

The computer-assisted two-dimensional gel electrophoresis methodology developed within this laboratory has provided a highly sensitive procedure capable of resolving large numbers of proteins within a sample. This technique has been successfully applied to the investigation of differences in gene expression between normal, preneoplastic and neoplastic tissues. These studies have demonstrated qualitative as well as quantitative alterations in protein profiles. Of particular interest are those proteins which are produced in preneoplastic and neoplastic tissue but not in normal tissue. We have focused our studies on the differences in protein patterns obtained from 2D-PAGE analysis of Solt-Farber induced neoplastic hepatic nodules with the patterns obtained from analysis of normal hepatic tissue. At least four protein spots have been detected in neoplastic tissue which were not found in normal tissue (mol. wt., pI, location): (A) 57 kD, 6.8, cytosol; (B) 41 kD, 6.25, membrane; (C) 26 kD, 6.75, membrane; (D) 24 kD, 6.05, membrane.

The isolation and characterization of these proteins requires the development of a battery of microanalytical techniques due to the small quantity of protein present in a two-dimensional gel spot. Initial investigations have been focused on the development of microscale procedures for sample handling, enzymic digestion and peptide purification. In addition, classical procedures for peptide and protein sequencing involving Edman degradation cannot be applied to proteins and peptides whose amino-terminus is blocked and are also not applicable for the analysis of trace amounts of sample. Therefore, novel microsequencing techniques are being investigated.

Initial studies concerning the electroelution of I-125 labeled proteins from polyacrylamide gels have demonstrated a poor recovery of protein from silver stained gels. In contrast, protein elution following visualization with coomassie blue dye was successful, in that between 65% and 95% of the protein could be recovered depending on the protein used (albumin, carbonic anhydrase or trypsin inhibitor) and the amount applied to the gel (15-1000 pmoles). Amino acid analysis of the resulting protein solution has so far been unsuccessful due to contamination of the sample with amino acids and other components also eluted from the gel.

Methodology for the trypsin digestion of pmole amounts of proteins is currently being developed. Preliminary studies using carbonic anhydrase as a model protein have been successful and amino acid analysis of the tryptic peptides separated by reverse-phase HPLC was performed. Work is presently underway to modify the dipeptidase method of peptide sequencing using electron impact mass spectrometry devised by Krutzsch (1983) to allow sequencing of these trypic peptides.

5. Initiation and Termination of Hepatocyte Proliferation by Serum Factors

Previous studies in other laboratories have shown that serum obtained from partially hepatectomized rats is more active in stimulating DNA synthesis in primary hepatocyte cultures than serum obtained from control rats. We are investigating serum from hepatectomized rats in order to determine the components responsible for this difference. We have achieved a partial purification of a factor which stimulates DNA synthesis in primary cultures of rat hepatocytes using heparin-affinity chromatography and gel filtration of serum from Fischer rats 24 hours after two-thirds partial hepatectomy. A 6 to 7-fold increase in DNA synthesis compared to control was observed on addition of the partially purified serum factor (10 µl) to the primary hepatocyte cultures (100 µl). The stimulation was dose-dependent. A similar increase in DNA synthesis (6 to 7-fold) was observed on addition of EGF (10 ng/ml) plus insulin (0.1 µM) to the hepatocyte cultures. In contrast the growth factors PDGF and EGF produced no stimulation of DNA synthesis in these cell cultures. The partially purified serum factor has an apparent molecular weight of 70,000-120,000 daltons and is stable for at least two months at -70°C. Some activity is lost by treatment with acid at pH 4 followed by dialysis (10,000 mw-cutoff). This stimulatory factor is also active in cultures of normal rat kidney cells, but is not active in cultures of non-malignant liver cells isolated from a 12-day old male Fischer rat or in cultures of hepatoma cel lines. Similar results were obtained when EGF was tested in these cell cultures.

6. Guanosine Triphosphate Binding Site of Ras Protein by NMR and CD Spectroscopy

1. Using hydroxybenzotriazole esters of t-butoxycarbonyl-amino acids in the solid phase synthesis provides a more economical and efficient way for synthesizing peptides than the symmetric anhydride method which has been known to prevent racemization during the coupling reaction.

2. The data obtained from CD studies of both the glycine-containing (Gly-peptide) and the valine-containing (Val-peptide) 34 amino acid residue N-terminal sequence of p21 proteins indicates that they both adopt predominately the beta-sheet conformation (60-55%) in non-ionic detergent solution. The result suggests that the secondary structure of the N-terminal sequence of p21 proteins is most likely to adopt a beta-alpha-beta conformation predicted by Hol, but unlike Feldmann's model which predicts only an alpha-helix structure.

3. Upon addition of GTP to the peptide solution, both Gly- and Val-peptide conformations remained in the beta-sheet conformation. However, the random coil conformation gradually increased at the expense of beta-sheet conformation.

4. When the Gly-peptide was added to GTP solution containing sodium dodecyl sulfate, the line widths of the P-31 signals of both beta and gamma resonance of GTP were broadened significantly (about 10 Hz), implying that the binding probably occurs between GTP and the Gly-peptide.

The Hormone Action and Oncogenesis Section plans and develops laboratory research on the mechanisms of the regulation of gene expression by hormones and growth factors. The major efforts of the Section are focused on: (1) characterization of the regulation of mouse mammary tumor virus (MMTV) expression by glucocorticoid hormones using advanced recombinant DNA techniques; (2) developing methods for in vitro DNA mediated gene transfer; and (3) studies on mechanism of action of growth factors and other transformation related factors with particular emphasis on characterization of the cellular receptors.

1. Molecular Basis of Steroid Hormone Action

Regulatory signals involved in the control of MMTV transcription by glucocorticoids have been examined in detail in two independent expression systems. In the first, fusions between the v-ras gene of HaMuSV, the MMTV LTR, and an enhancer element from the HaMuSV LTR have allowed us to monitor steroid-inducible transcription from the MMTV LTR by a rapid transfection assay. Efficient transfection of NIH3T3 cells to the transformed phenotype occurs only when glucocorticoids are present in the medium. In the second assay system, the CAT from the bacterial Tn9 transposon has been placed under control of the MMTV promoter; in this system, the elaboration of CAT enzyme activity was shown to be inducible by glucocorticoids during transient expression after acute DNA-mediated transfection. Using these assays, deletion analysis by molecular techniques has localized sites sufficient to confer hormone sensitivity on the MMTV LTR to between 100 and 200 nucleotides 5' to the MMTV cap site.

In both of these assay systems, an increase in the uninduced, constitutive expression from the MMTV LTR was observed after deletion of the hormone responsive sequences, suggesting that the mechanism of hormone action may be more complex than a simple induction effect. It is now apparent that the glucocorticoid regulatory element can regulate the activity of an exogenous enhancer introduced

into the fusion chimeras. A model is suggested in which the hormone regulatory sequence is actually composed of more than one element, a positive activator sequence and another previously uncharacterized element that regulates the activity of the positive element.

A competition assay has been developed in which the interaction between glucocorticoid receptor protein and MMTV DNA containing the hormone target region can be detected. LTR sequences implicated in the hormone response by the gene transfer experiments described above have been shown to compete for receptor binding in receptor-containing cell-free extracts more efficiently than random DNA sequences. This assay serves as one measure of the interaction of the glucocorticoid receptor and its target. The "oligo-scanning" mutagenesis system has been constructed so that mutants identified by the biological response as containing lesions in critical sequences can be amplified and tested easily in this competition assay.

2. Hormone-dependent Transcriptional Regulatory Elements: Structure and Function

A new technology has been derived for the rapid isolation of extensively mismatched mutants in regions of interest. Evidence from physical characterization of nucleoprotein structure at the hormone-regulated MMTV promoter indicates that chromatin is highly organized in this region. Deletion mutagenesis thus suffers a serious weakness in that large-scale removal of sequences probably alters several parameters of structure simultaneously. Alternatively, single-base mutants usually do not impair the structure sufficiently to score a clear phenotype in the test system.

In the new methodology, called "oligo-scanning mutagenesis," the v-ras MMTV fusion system has been transferred into the M13 bacteriophage, permitting high-resolution site-directed mutagenesis in gapped molecules created by heteroduplex formation between single-stranded molecules containing complete LTR regions and duplex molecules deleted for selected areas of the regulatory sequence. The test system after transfer to the M13 vector remains highly inducible to the action of glucocorticoid hormones, manifesting a 200-fold response in the number of foci recovered in the presence of hormone compared to the absence of hormone. Furthermore, S1 nuclease analysis indicates that transcription is initiated at the correct cap site in the test molecules, and that intracellular RNA levels respond normally to hormone regulation.

The new technique permits the simultaneous change of as many as 10 base pairs without changing the relative position of non-mutated sequences with respect to each other, or with respect to other elements of the promoter.

Results obtained with a preliminary set of mutant combinations indicates that (1) elimination of individual binding sites for the receptor complex leads to only partial impairment of the hormone response, and (2) major disruption of the hormone response region results in a large increase in the constitutive level of transcription in the absence of hormone. Therefore, the negative effect on transcription associated with the MMTV LTR by earlier deletion experiments has now been shown to be mediated through the same set of sequences that encode the receptor-binding sites. A long-range, high-resolution analysis of this region to characterize the mechanism of the positive and negative aspects of hormone regulation is underway.

3. Transmission of Mammalian Genes with Expression-regulated Retrovirus Vectors

A series of retroviral transmission vectors have been constructed which utilize the LTRs and associated replication functions from MuLV for transmission of the vector, and make use of the glucocorticoid-regulated promoter from MMTV for regulated expression. Transcriptional enhancer elements from a variety of sources are also incorporated as accessory elements to modulate the strength of the MMTV promoter.

Two questions have been addressed. First, functional expression of a retroviral convected gene cassette has been separated from transmission by encoding the information to be expressed on the opposite strand, in the antiparallel sense, and providing it with a promoter separate from the transmission LTRs. Thus functional cassettes that include polyadenylation signals and splicing signals can be transmitted without the problems associated with RNA processing that occurs at these signals when they are included on the transmission strand.

Secondly, we have shown that the ras oncogene can be transmitted into cells with these vectors under conditions where its expression is conditional. That is, when the oncogene is driven from the MMTV promoter in either the parallel or antiparallel configuration, transformation of the cell is dependent on the presence of glucocorticoids.

4. Chromatin Structure and Steroid Hormone Action

Nucleosome positioning on the LTR of mouse mammary tumor virus, which encodes a glucocorticoid dependent promoter, has been found to be sequence specific. An array of phased nucleosomes exists upstream of the initiation site for MMTV transcription, beginning at position -70, and extending through to the left end of the LTR. The first nucleosome in this phased array includes sequences which are necessary for transfer of biological regulation in gene transfer experiments, and which contain two binding sites for glucocorticoid receptor in vitro. The sequences which interact with the hormone regulatory molecule are, therefore, displayed on the surface of a phased nucleosome. This same region becomes hypersensitive to DNase I upon activation of the promoter with hormone, indicating a major alteration in chromatin structure in this regulatory region.

In addition to their intramural research efforts that have been summarized above, investigators within the LEC serve on editorial boards of major journals in their field, and are involved as consultants or advisors on various national and international committees in the area of chemical and biological carcinogenesis. Furthermore, the LEC scientists participate to a considerable degree in collaborative efforts with scientists both within the NCI and throughout the country, and the international scientific community.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP04986-08 LEC

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Molecular Basis of Steroid Hormone Action

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Gordon L. Hager	Head, Hormone Action & Oncogenesis Section	LEC	NCI
Others:	Ronald G. Wolford	Microbiologist	LEC	NCI
	Diana S. Berard	Microbiologist	LEC	NCI

COOPERATING UNITS *(if any)*

Laboratory of Chemistry, NIADDK, NIH (S. Simmons)

LAB/BRANCH

Laboratory of Experimental Carcinogenesis

SECTION

Hormone Action and Oncogenesis Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.4	0.6	0.8

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Previous analysis of molecular chimeras between the mouse mammary tumor virus (MMTV) long terminal repeat (LTR) and the v-ras transformation gene from Harvey murine sarcoma virus (HaMuSV), and between the LTR and the chloramphenicol acetyl transferase (CAT) gene localized the steroid hormone regulatory sequences between 100 and 200 nucleotides upstream from the cap site in the LTR. Utilization of a competition assay with specific MMTV-LTR fragments and total cellular DNA immobilized on cellulose has shown the preferential binding of the glucocorticoid receptor to fragments of LTR DNA containing the sequences identified in gene transfer experiments as important for hormone regulation. The addition of transcriptional activator sequences to the MMTV promoter indicates that the hormone regulatory sequence is capable of regulating the activity of the exogenous enhancer. These observations suggest a model for the mechanism of hormone action in which the regulatory sequence acts as a modulator of another cis-dominant positive element.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

Gordon L. Hager	Head, Hormone Action & Oncogenesis Section	LEC	NCI
Ronald G. Wolford	Microbiologist	LEC	NCI
Diana S. Berard	Microbiologist	LEC	NCI

Objectives:

(1) Analysis of hormone regulated transcription of mouse mammary tumor virus (MMTV).

(2) Determination of the mechanism of hormone action in the up-regulated MMTV system; extension of the investigation of glucocorticoid regulation into systems where the expression is down-regulated.

(3) Application of tools developed for the study of hormone action to the study of other transcriptional regulatory systems important in cell growth.

Methods Employed:

Molecular chimeras between the MMTV long terminal repeat (LTR) and the v-ras gene of Harvey murine sarcoma virus (HaMuSV) are used in a hormone-dependent transfection assay to probe the regulatory regions involved in hormone induction of MMTV expression. Similar fusions between the LTR and the chloramphenicol acetyl transferase (CAT) gene from the bacterial Tn9 transposon will be tested in a transient expression assay. Deletion analysis of molecular chimeras will be performed to localize these regions.

Utilizing the S1 nuclease or mung bean nuclease mapping techniques, probes available from molecular clones of MMTV will be utilized to analyze steroid-dependent MMTV regulation.

Transcriptional promoters with negative response to glucocorticoids (proopiomelanocortin), but responsive to other hormones (androgens) will be engineered into the v-ras transformation system to test for transfer of regulation.

Initiation of transcription at the regulated MMTV promoter will be carried out in Xenopus oocytes. An analysis of the role of chromatin organization in hormone action will be carried out by comparing naked DNA templates and minichromosomes containing MMTV promoters as transcription templates in this system.

Minichromosomes containing the MMTV promoter mobilized on the bovine papilloma virus episomal vector (see project # Z01CP05450-01) will serve as a nucleoprotein template for in vitro transcription experiments. Various nuclear extracts and purified glucocorticoid receptor will also be used in these transcription experiments. The cloned, naked DNA is used as a control.

Major Findings:

Regulatory signals involved in the control of MMTV transcription by glucocorticoids have been examined in detail in two independent expression systems. In the first, fusions between the v-ras gene of HaMuSV, the MMTV LTR, and an enhancer element from the HaMuSV LTR have allowed us to monitor steroid-inducible transcription from the MMTV LTR by a rapid transfection assay. Efficient transfection of NIH 3T3 cells to the transformed phenotype occurs only when glucocorticoids are present in the medium. In the second assay system, the CAT from the bacterial Tn9 transposon has been placed under control of the MMTV promoter; in this system, the elaboration of CAT enzyme activity was shown to be inducible by glucocorticoids during transient expression after acute DNA-mediated transfection. Using these assays, deletion analysis by molecular techniques has localized sites sufficient to confer hormone sensitivity on the MMTV LTR to between 100 and 200 nucleotides 5' to the MMTV cap site.

In both of these assay systems, an increase in the uninduced, constitutive expression from the MMTV LTR was observed after deletion of the hormone responsive sequences, suggesting that the mechanism of hormone action may be more complex than a simple induction effect. It is now apparent that the glucocorticoid regulatory element can regulate the activity of an exogenous enhancer introduced into the fusion chimeras. A model is suggested in which the hormone regulatory sequence is actually composed of more than one element, a positive activator sequence, and another previously uncharacterized element that regulates the activity of the positive element.

A competition assay has been developed in which the interaction between glucocorticoid receptor protein and MMTV DNA containing the hormone target region can be detected. LTR sequences implicated in the hormone response by the gene transfer experiments described above have been shown to compete for receptor binding in receptor-containing cell-free extracts more efficiently than random DNA sequences. This assay serves as one measure of the interaction of the glucocorticoid receptor and its target. The "oligo-scanning" mutagenesis system (see Project No. Z01CP05378-02) has been constructed so that mutants identified by the biological response as containing lesions in critical sequences can be amplified and tested easily in this competition assay.

Significance to Biomedical Research and the Program of the Institute:

A major goal of the experimental program is to decipher the mechanisms involved in steroid hormone regulation as a model for the general problem of hormone action. Control of cell proliferation is a central issue in neoplastic transformation; hormones are directly implicated in many aspects of growth control. Steroid hormones are directly implicated in the biology of certain human neoplasms, such as breast cancer. A thorough knowledge of the mechanisms of hormone action is therefore necessary to our eventual understanding and control of the neoplastic process.

Proposed Course:

High-resolution mutants in the hormone response region will be examined in the *Xenopus oocyte* transcription system to determine their effect on the basic

promoter structure of the MMTV LTR. Efforts will also be made to demonstrate regulation in the oocyte system by co-injection of extracts enriched in glucocorticoid receptors.

The role of chromatin structure in the hormone response will be investigated in the oocyte system by comparing the transcription initiation capacity of naked DNA templates and minichromosomes isolated from cells harboring bovine papilloma virus (BPV) MMTV LTR episomal chimeras.

Attempts will be continued to demonstrate correct initiation in cell-free transcription extracts, and to demonstrate regulation with receptor-enriched extracts, DNA and minichromosomal templates.

A further extrapolation of the tools developed for the study of hormone action in the up-regulated MMTV system will be carried into the study of the down-regulated pro-opiomelanocortin (POMC) complex. Chimeric molecules with the induced MMTV promoter and the repressed POMC promoter will be constructed on the BPV episomal vector.

Publications:

Miller, P. A., Ostrowski, M. C., Hager, G. L. and Simons, S. S., Jr.: Covalent and non-covalent receptor-glucocorticoid complexes preferentially bind to the same regions of the long terminal repeat of murine mammary tumor virus proviral DNA. Biochemistry 23: 6883-6889, 1984.

Ostrowski, M. C., Huang, A. L., Kessel, M., Wolford, R. G. and Hager, G. L.: Modulation of enhancer activity by the hormone responsive regulatory element from mouse mammary tumor virus. EMBO J. 3: 1891-1899, 1984.

Slagle, B. L., Wheeler, D. A., Hager, G. L., Medina, D. and Butel, J. S.: Molecular basis of altered mouse mammary tumor virus expression in the D2 hyperplastic alveolar nodule line of Balb/C mice. Virology 143: 1-15, 1985.

Weeks, M. O., Hager, G. L., Lowe, R. and Scolnick, E. M.: Development and analysis of a transformation-defective mutant of Harvey murine sarcoma tk virus and its gene product. J. Virol. 54: 586-597, 1985.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05260-04 LEC

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Regulation of Gene Expression and Differentiation in Neoplasia

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	Carole A. Heilman	Senior Staff Fellow	LEC	NCI
Others:	Snorri S. Thorgeirsson	Chief	LEC	NCI
	Irene B. Glowinski	Staff Fellow	LEC	NCI
	Nancy Kim	Chemist	LEC	NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Experimental Carcinogenesis

SECTION

Chemical Carcinogenesis Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.7	0.7	1.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The object of this project is to examine the regulation of gene expression associated with cellular differentiation in two experimental model systems; 1) the human promyelocytic leukemia cell line, HL60, and 2) the rat hepatoma cell lines, Reuber and 7777. The results obtained in the HL60 cell line study include a functional association of c-myc RNA levels with the differentiation state of the cell. Specifically, expression of c-myc is almost exclusively associated with the promyelocytic status of the HL60 cells and this expression is repressed during differentiation of the promyelocytes to monocytes. Conversely N-ras specific transcription is effe-ted to no significant extent during differentiation specific or growth specific treatment. The results obtained and experiments in progress in the rat hepatoma cell study include an albumin specific gene response following treatment with butyric acid or inhibitors of ADP-ribosylation. This response was independent of the "differentiation state" of the cells, as measured by alpha-fetoprotein (AFP) or albumin production, since the albumin specific response was present in the high albumin, high AFP producer cell line (Reuber) or the low albumin, low AFP producer line (7777). The AP gene was not affected by this treatment protocol in either cell line.

PHS 6040 (Rev. 1/84)

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

Carole A. Heilman	Senior Staff Fellow	LEC	NCI
Snorri S. Thorgeirsson	Chief	LEC	NCI
Irene B. Glowinski	Staff Fellow	LEC	NCI
Nancy Kim	Chemist	LEC	NCI

Objectives:

The objective of this project is to examine the regulation genes associated with differentiation by employing molecular biology and biochemical techniques. Our aim is to identify and understand both cellular and genetic factors that are important in differentiation. The experimental systems which we have under investigation include (1) the human promyelocytic leukemia cell line, HL60 and (2) the rat hepatoma cell lines, Reuber and 7777.

Methods Employed:

Methods used in these studies include: tissue culture techniques; radioimmunoassay; differentiated centrifugation and chromatographic techniques; radioisotopic measurements using tritium, carbon-14, phosphorus-32 and iodine-125; enzyme assays involving radiometric or spectrophotometric determination; and recombinant and molecular technology including DNA and RNA preparations, Northern and Southern blotting, and nucleic acid hybridization.

Major Findings:

A. Studies on the Human Promyelocytic Leukemia Cell Line, HL60: Independent Expression of c-myc and N-ras during Growth and Differentiation. The HL60 cell line, a continuously proliferating suspension cell culture originally derived from a patient with acute promyelocytic leukemia is widely used as an in vitro model for studying cellular differentiation along the myeloid/monocyte pathways. Treatment of HL60 cells with compounds such as retinoic acid, hypoxanthine, actinomycin D, butyrate, dimethyl sulfoxide and hexamethylene bisacetamide results in a greater than 90% commitment of the cells to differentiate, both functionally and morphologically, into mature granulocytes. Exposure of HL60 cells to phorbol esters, such as 12-O-tetradecanoylphorbol-13-acetate (TPA), results in an irreversible commitment to monocyte differentiation, characterized by the shift from suspension to adherent cells, the acquisition of macrophage associated surface markers, monocyte specific esterases and the cessation of cell growth associated with terminal differentiation.

Treatment with HL60 cells with difluoromethyl ornithine (DFMO), an irreversible inhibitor of ornithine decarboxylase (ODC), results in a cessation of growth without committing the cells to differentiate. However, DFMO does not prevent differentiation of HL60 cells into monocytes when TPA is subsequently added, thus demonstrating that TPA-induced monocytic differentiation is independent of the decrease in cell proliferation also associated with TPA treatment.

We investigated the expression of two of the HL60 associated oncogenes, c-myc and N-ras, both during terminal differentiation and growth inhibition by DFMO that is independent of terminal differentiation. The c-myc oncogene is present in multiple copies in this cell line as well as in the original cell isolate and is highly responsive to treatment with retinoic acid of dimethylsulfoxide. C-myc transcripts are reduced 80-90% in HL60 cells treated with these granulocyte differentiating compounds when compared to untreated controls. The N-ras oncogene has been reported to be the transformation specific gene in HL60 as assayed by the transfection of NIH3T3 cells.

We have associated the 8-10 fold decrease in c-myc transcripts with differentiation of the promyelocytes into mature monocytes. C-myc regulation also appears to be an early event of HL60 cells as evidenced by a burst of c-myc synthesis within the first hour of TPA treatment, followed by the rapid decline in the level of c-myc specific transcripts. This response was similar to that observed in mitogen stimulated B cells, T cells, and normal fibroblasts although the HL60 induced level was neither as dramatic nor as long as that reported for normal cells. Although this response was reportedly associated with the cell cycle regulation of proliferation in normal cells, the level of c-myc specific transcripts was found invariant during the HL60 cell cycle. In addition, unlike the mitogenic response of other agents, or normal resting cells, TPA induced differentiation of HL60 cells resulted in no cellular proliferation or significant change in DNA synthesis when compared to control or DFMO treated cells during the first 24 hours, again supporting the association of c-myc with the diffentiation state of the cell.

(B) Modulation of Albumin Gene Expression in the Rat Hepatoma Lines, Reuber and 7777, by Sodium Butyrate and ADP-ribosylation. Chromatin conformation plays a key role in the regulation of gene expression. The use of chemical compounds which alter the chromatin structure has helped to elucidate the relationship between conformational modifications and gene expression. A strong correlation between transcriptionally active chromatin and acetylated histones has been reported in a number of eukaryotic systems following treatment with sodium butyrate (BA). However, there is equally strong evidence for the inactivation of genes, specifically the hormonally induced gene expression of ovalalbumin and transferrin in oviduct explants and tyrosine aminotransferase in HTC cells following treatment with BA. Thus, although histone hyperacetylation is associated with the regulation of gene expression its functional specificity and relationship to other controlling elements is presently unclear.

The involvement of poly ADP-ribosylation of histone proteins in the regulation of gene expression has also been studied in several cell systems using two inhibitors of poly ADP-ribosyl transferases (ADPRT's), nicotinamide and 3-aminobenzamide (3-AB). For example, in the 341 mouse carcinoma cell line, the glucocorticoid inducible expression of mouse mammary tumor virus was stimulated when cells were incubated in the presence of 3-AB. In contrast, the induction of two oncofetal proteins following in vitro culturing of primary adult rat hepatocytes, γ-glutamyl transpeptidase and the K-type III isoenzyme of pyruvate kinase was inhibited when cells were incubated in the presence of 3-AB or nicotinamide. Thus, although the modification of histone and nonhistone proteins by poly ADP-ribose appears to effect the expression of certain genes, the synergistic effect of other positive and negative regulators appears to be important.

To further understand the relationship between chemically-induced conformational changes of chromatin and the expression of specific genes, the effects of BA and inhibitors of poly ADPRT on two liver specific genes that are associated with both normal differentiated hepatocytes (i.e., albumin gene) and the oncofetal stage (i.e. α-fetoprotein (AFP) gene) were investigated in the Reuber rat hepatoma line, H4-II-E, and in the 7777 rat hepatoma line. These two cell lines differ in that the albumin gene is expressed in high quantities in the Reuber cell line and low quantities in the 7777 cell line while AFP is expressed in low quantities in the Reuber cell line and high quantities in the 7777 cell line. We have demonstrated that the albumin gene, in both cell lines, was specifically affected by the treatment with BA and inhibitors of ADPRT. In the Reuber cell line, a 55-fold increase in albumin secretion and an approximate 10-fold increase in the level of albumin specific RNA was demonstrated following treatment with 3 mM BA for 72 hours. Unlike the inhibitory effect of BA on the induction of tyrosine aminotransferase activity by dexamethasone in HTC cells, the effect of BA on albumin synthesis in Reuber cells was not rapid (hours vs minutes) showing no change in albumin secretion for up to a 3 hour post-treatment period, when compared to untreated controls, and reaching a maximal role of albumin secretion of 72 hours post-treatment. No affect on the levels of AFP specific RNA was observed in either cell line following treatment with BA.

The effect of inhibitors of ADP-ribosylation was next determined in cells maintained in the presence or absence of BA. Treatment of Reuber cells with 3 mM 3-aminobenzoic acid (3-AB) dramatically reduced the level of albumin specific RNA. A similar decrease was also observed following treatment with 10 mM nicotinamide, another inhibitor of poly ADPRT. Incubation in the presence of the non-inhibitory analogue, meta-aminobenzoic acid at 3 mM did not affect the levels of albumin specific RNA. Interestingly, the inhibitory effect of 3-AB on albumin RNA levels was overcome to some extent when cells were coincubated in the presence of both 3-AB and BA. These results were also reflected in albumin secretion, as measured by radioimmunoassay. BA also appeared to have a direct positive effect on the levels of poly ADP-ribosylation in Reuber cells when assayed using the in vitro nuclear system in the presence of 3 M NAD. No effect on the AFP specific RNA was demonstrated under this treatment protocol.

Thus, we have a system in which a "differentiation" specific gene is regulated by two chemical modulators of chromatin, BA and inhibitors of only ADP-ribosylation. This effect is seen in two cell lines whose base level of albumin specific RNA are very different, suggesting that the effect of BA and inhibitors of poly ADP-ribosylation are acting specifically on this differentiation associated gene rather than on a gene which is normally "turned on." We are presently trying to determine if treatment with BA has any direct effect on the DNAse I sensitivity patterns for the albumin gene vs the AFP genes, thus giving additional information concerning the specific interactions of BA on chromatin.

Significance to Biomedical Research and the Program of the Institute:

Our studies are aimed at identifying and characterizing both the cellular and genetic factors important in chemically-induced and spontaneous neoplasia. The information obtained from these studies could provide a basis for a better definition of the factors involved in cancer cause and may help in formulating an effective cancer prevention program.

Proposed Course:

Continue the course outlined under Objectives and Major Findings.

Publications:

Huber, B., Dearfield, K. L., Williams, J. R., Heilman, C. A. and Thorgeirsson, S. S.: The tumorigenicity and transcriptional modulation of c-myc and N-ras oncogenes in a human hepatoma cell line. Cancer Res. (In Press)

Kessler, D. J. Heilman, C. A., Cossman, J., Maguire, R. T. and Thorgeirsson, S. S.: Chemical transformation of EBV immortalized human B cells. Cancer Res. (In Press)

Thorgeirsson, U. P., Turpeenniemi-Hujanen, T., Williams, J. E., Westin, E. H., Heilman, C. A., Talmadge, J. E. and Liotta, L. A.: NIH/3T3 cells transfected with human tumor DNA containing activated ras oncogenes express the metastatic phenotype in nude mice. Mol. Cell. Biol. 5: 259-262, 1985.

Tosato, G., Steinberg, A. D., Yarchoan, R., Heilman, C. A., Pike, S. E., De Seau, V. and Blaese, R. M.: Abnormally elevated frequency of Epstein-Barr virus-infected B cells in the blood of patients with rheumatoid arthritis. J. Clin. Invest. 73: 1789-1795, 1984.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01CP05261-04 LEC

PERIOD COVERED
October 1, 1984 to September 30, 1985

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)
Metabolism and Mutagenicity of Chemical Carcinogens

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

	Name	Title	Lab	Institute
PI:	Snorri S. Thorgeirsson	Chief	LEC	NCI
Others:	Irene B. Glowinski	Staff Fellow	LEC	NCI
	Ritva P. Evarts	Veterinary Medical Officer	LEC	NCI
	Peter J. Wirth	Expert	LEC	NCI
	Preston H. Grantham	Chemist	LEC	NCI
	Ronald F. Minchin	Visiting Associate	LETM	NCI

COOPERATING UNITS (if any)
National Institute of Public Health, Oslo, Norway (E. Dybing); Scripps Clinic and Research Foundation, La Jolla, CA (E. Johnson)

LAB/BRANCH
Laboratory of Experimental Carcinogenesis

SECTION
Chemical Carcinogenesis Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.3	1.3	0

CHECK APPROPRIATE BOX(ES)
☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The research is at present focused on the relative roles of metabolic activation and detoxification in determining both mutagenic and carcinogenic potential of aromatic amines and amides. Results so far obtained include: (1) Two distinct phenotypes, slow and fast metabolizers, were observed for both metabolic activation and detoxification of the model chemical carcinogen, 2-acetylaminofluorene, in human liver microsomes from 28 individuals. We observed that individuals who were fast activators of the carcinogen were, in most cases, also fast detoxifiers of the chemical. However, different phenotype patterns exist suggesting that fast activators of a toxin need not also be phenotyped as fast detoxifiers. Studies in this area may help understand whether certain individuals are predisposed to a higher rate of chemically-induced cancers. (2) The kinetics of cytochrome P-450 dependent N-hydroxylation of 2-aminofluorene (AF) and 4-aminobiphenyl (4-AB) were determined using six highly purified forms of rabbit cytochrome P-450 and microsomes from control and 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) induced rabbit liver and lung. N-Hydroxylation of both AF and 4-AF was best defined by two enzyme systems, showing a high affinity low capacity and a low affinity high capacity, in control and TCDD microsomes. Pretreatment of TCDD modified the apparent Km and Vmax for the N-hydroxylation of both substrates in liver and lung microsomes, but the biphasic kinetics were observed in all instances. Form 4 was the only form capable of catalyzing the N-hydroxylation of AF and 4-AB. The kinetic data obtained with form 4 for these two substrates were consistent with a single enzyme system. α-Naphthoflavone (ANF) completely inhibited the N-hydroxylation of AF in liver microsomes from both control and TCDD treated animals, whereas only partial inhibition was obtained with lung microsomes. These results indicate that at least two forms of cytochrome P-450 with greatly different substrate affinity (Km) participate in the N-hydroxylation of primary aromatic amines in rabbit liver and lung microsomes.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

Snorri S. Thorgeirsson	Chief	LEC	NCI
Irene B. Glowinski	Staff Fellow	LEC	NCI
Ritva P. Evarts	Veterinary Medical Officer	LEC	NCI
Peter J. Wirth	Expert	LEC	NCI
Preston H. Grantham	Chemist	LEC	NCI
Ronald F. Minchin	Visiting Associate	LETM	NCI

Objectives:

The main objectives of the project are (1) to define, in an intact cell system, the metabolic processing of chemical carcinogens, especially carcinogenic aromatic amines and amides, and to identify the metabolic pathways that are responsible for activation and detoxification of these compounds; (2) to study the mechanism whereby carcinogenic aromatic amines and amides cause mutations and other types of genotoxicity in both microbial and mammalian cell systems; and (3) to characterize the kinetic parameters for both metabolic activation and detoxification reactions for carcinogenic aromatic amines.

Methods Employed:

The principal methods are (1) bacterial and mammalian culture techniques, (2) differential centrifugation, (3) enzyme assays, (4) recording spectrophotometry, and (5) high pressure liquid chromatography.

Major Findings:

Cytochrome P-450 Dependent Metabolism. 1. Studies with Human Tissues. Two distinct phenotypes, slow and fast metabolizers, were observed for both metabolic activation and detoxification of the model chemical carcinogen, 2-acetylaminofluorene, in human liver microsomes from 28 individuals. We observed that individuals who were fast activators of the carcinogen were, in most cases, also fast detoxifiers of the chemical. However, different phenotype patterns exist suggesting that fast activators of a toxin need not also be phenotyped as fast detoxifiers. Studies in this area may help understand whether certain individuals are predisposed to a higher rate of chemically-induced cancer.

2. Studies with Animal Tissues. (a) The kinetics of cytochrome P-450 dependent N-hydroxylation of 2-aminofluorene (AF) and 4-aminobiphenyl (4-AB) were determined using six highly purified forms of rabbit cytochrome P-450 and microsomes from control and 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) induced rabbit liver and lung. N-Hydroxylation of both AF and 4-AB was best defined by two enzymes systems, showing a high affinity low capacity and a low affinity high capacity, in control and TCDD microsomes. Pretreatment with TCDD modified the apparent Km and Vmax for the N-hydroxylation of both substrates in liver and lung microsomes, but the biphasic kinetics were observed in all instances. Form 4 was the only form capable of catalyzing the N-hydroxylation of AF and 4-AB. The kinetic data obtained with

form 4 for these two substrates were consistent with a single enzyme system. α-Naphthoflavone (ANF) completely inhibited the N-hydroxylation of AF in liver microsomes from both control and TCDD treated animals, whereas only partial inhibition was obtained with lung microsomes. These results indicate that at least two forms of cytochrome P-450 with greatly different substrate affinity (Km) participate in the N-hydroxylation of primary aromatic amines in rabbit liver and lung microsomes.

(b) The metabolism of 2-acetylaminofluorene (AAF) has been used to study cytochrome P-450 monooxygenase activity in two rat hepatoma cell lines, McA-RH7777 and Reuber H4-II-E. McA-RH7777 cells exhibited considerably higher basal activities than H4-II-E cells for all metabolic pathways studied. Differences varied from 1.8-fold for the 9 hydroxylation to 11-fold for the 3- and 5-hydroxylations of AAF. Both phenobarbital and TCDD caused a marked induction of AAF metabolite formation in both cell lines. However, because of the low basal activities of AAF metabolite formation in H4-II-E cells, the fold induction caused by phenobarbital in these cells still results in activities below those of control McA-RH7777 cells. The half-life of AAF disappearance in control, phenobarbital and TCDD pretreated McA-RH7777 cells were 240, 34 and 23 min, respectively. This is the first time the cytochrome P-450 monooxygenase system of an established cell line has been shown to respond equally to both phenobarbital and TCDD induction.

Significance to Biomedical Research and the Program of the Institute:

Our studies are aimed at providing a better understanding of the metabolic processes that determine activation and/or detoxification of procarcinogens. We are also studying the mechanism whereby chemical carcinogens exert their genotoxic effects in both microbial and mammalian cell systems. The information derived from these studies may provide a sounder basis for possible prevention (chemoprevention) of chemically induced tumors as well as for identifying individuals at risk to develop cancer.

Proposed Course:

The scope of this project has been significantly reduced, and will focus primarily on the metabolism and metabolic activation of the mutagenic and carcinogenic heterocyclic amines found in pyrolysates of amino acids and proteins or isolated from broiled fish or beef.

Publications:

Boobis, A. R., Murray, S., Hampden, C. E., Harris, G. C., Huggett, A. C., Thorgeirsson, S. S., McManus, M. E. and Davies, D. J.: Enzymic basis for polymorphism of drug oxidation in man. In Boobis, A. R., Caldwell, J., DeMatteis, F., and Elcombe, C. E. (Eds.): Microsomes and Drug Oxidations. London, Taylor & Francis, Ltd., 1985, pp. 361-369.

Glowinski, I. B., Sanderson, N. D., Hayashi, S. and Thorgeirsson, S. S.: Metabolic activation and genotoxicity of N-hydroxy-2-acetylaminofluorene and N-hydroxyphenacetin derivatives in Reuber (H4-II-E) hepatoma cells. Cancer Res. 44: 1098-1104, 1984.

Hayashi, S., Moller, M. and Thorgeirsson, S. S.: Genotoxicity of heterocyclic amines in the Salmonella/hepatocyte system. Jpn. J. Cancer Res. (Gann) (In Press)

Mattison, D. R., Chang, L., Thorgeirsson, S. S. and Shiromizu, K.: The effects of cyclophosphamide, azathioprine, and 6-mercaptopurine on oocyte and follicle number in C57BL/6N mice. Fertil. Steril. (In Press)

Mattison, D. R., Shiromizu, K., Pendergrass, J. R. and Thorgeirsson, S. S.: Ontogeny of ovarian glutathione and sensitivity to primordial oocyte destruction by cyclophosphamide. Pediat. Pharmac. 3: 49-55, 1983.

McManus, M. E., Boobis, A. R., Minchin, R. F., Schwartz, D.M., Murray, S., Davies, D. S. and Thorgeirsson, S. S.: Relationship between oxidative metabolism of 2-acetylaminofluorene, debrisoquine, bufuralol and aldrin in human liver microsomes. Cancer Res. 44: 5692-5697, 1984.

McManus, M. E., Minchin, R. F., Sanderson, N., Schwartz, D., Johnson, E. F., and Thorgeirsson, S. S.: Metabolic processing of 2-acetylaminofluorene by microsomes and six highly purified cytochrome P450 forms from rabbit liver. Carcinogenesis 5: 1717-1723, 1984.

Minchin, R. F., McManus, M. E., Thorgeirsson, S. S., Schwartz, D., and Boyd, M. R.: Metabolism of 2-acetylaminofluorene in isolated rabbit pulmonary cells: Evidence for the heterogeneous distribution of monooxygenase activity in lung tissue. Drug Metab. Dispos. (In Press)

Moller, M., Glowinski, I. B. and Thorgeirsson, S. S.: The genotoxicity of aromatic amines in primary hepatocytes isolated from C57BL/6 and DBA/2 mice. Carcinogenesis 5: 797-804, 1984.

Moller, M. and Thorgeirsson, S. S.: DNA damage induced by nitropyrenes in primary mouse hepatocytes and in rat H4-II-E hepatoma cells. Mutation Res. (In Press)

Razzouk, C., McManus, M. E., Hayashi, S., Schwartz, D. and Thorgeirsson, S. S.: Induction of epoxide hydrolase in cultured rat hepatocytes and hepatoma cell lines. Biochem. Pharmacol. 34: 1537-1542, 1985.

Shiromizu, K., Thorgeirsson, S. S. and Mattison, D. R.: The effect of cyclophosphamide on oocyte and follicle number in Sprague-Dawley rats, C57BL/6N and DBA/2N mice. Pediat. Pharmac. 4: 213-221, 1984.

Thorgeirsson, S. S.: Kinetics of 2-acetylaminofluorene hydroxylation reactions. In Boobis, A. R., Caldwell, J., DeMatteis, F. and Elcombe, C. E. (Eds.): Microsomes and Drug Oxidations. London, Taylor & Francis, Ltd., 1985, pp. 320-329.

Thorgeirsson, S. S. and Sato, S.: Meeting report - U.S./Japan Cooperative Cancer Research Program Seminar on Carcinogenicity, Mutagenicity and Metabolism of Heterocyclic Amines. Cancer Res. 45: 1908-1911, 1985.

Vu, V. T., Grantham, P. H., Roller, P. P., Hankins, W. D., Wirth, P. J., and Thorgeirsson, S. S.: Formation of DNA adducts from N-acetoxy-2-acetylamino-fluorene and N-hydroxy-2-acetylaminofluorene in rat hemopoietic tissues in vivo. Cancer Res. (In Press)

Vu, V. T., Moller, M. E., Grantham, P. H., Wirth, P. J. and Thorgeirsson, S. S.: Association between DNA strand breaks and specific DNA adducts in murine hepatocytes following in vivo and in vitro exposure to N-hydroxy-2-acetylaminofluorene and N-acetoxy-2-acetylaminofluorene. Carcinogenesis 6: 45-52, 1985.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05262-04 LEC

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Cellular Evolution of Chemically Induced Murine Hepatomas

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	Ritva P. Evarts	Veterinary Medical Officer	LEC	NCI
Others:	Snorri S. Thorgeirsson	Chief	LEC	NCI
	Elizabeth R. Marsden	Biologist	LEC	NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Experimental Carcinogenesis

SECTION

Chemical Carcinogenesis Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.9	0.0	1.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The goal of this project is to determine the sequence of cellular events that evolve during development of chemically induced murine hepatomas. In order to identify the cellular changes occurring during initiation, promotion and progression of neoplastic transformation, we have employed histochemical, immunohistochemical, radioreceptor and in situ hybridization techniques. Transplantation of hepatocytes to the anterior chamber of the rat eye is used to evaluate the stage of transformation and the proliferative capacity of hepatocytes. Results obtained include: (1) Preneoplastic liver cells were selected on the basis of their lack of affinity to tissue culture plates coated with asialofetuin; these preneoplastic cells showed a definite growth advanatage in the anterior chamber when promoted by phenobarbital. (2) Normal hepatocytes and hepatocytes treated with carcinogens survived in the anterior chamber for several months. (3) Cells derived from hyperplastic liver nodules progress into frank malignant hepatoma when transplanted to the anterior chamber of the eye. (4) Both phenobarbital, a liver specific tumor promoter, and partial hepatectomy reduced the number of liver-specific asialoglycoprotein receptors (ASGP-R) on the surface of the hepatocytes. The demonstrated lack of ASGP-R may be of importance as a mechanism of tumor promotion in the liver. (5) In situ hybridization of rat albumin and alpha-fetoprotein c-DNA probes for liver m-RNA has been used to define the conditions for in situ hybridization of c-DNA probes to target mRNA.

PHS 6040 (Rev. 1/84)

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

Ritva P. Evarts	Veterinary Medical Officer	LEC	NCI
Elizabeth R. Marsden	Biologist	LEC	NCI

Objectives:

The object of this project is to characterize the cellular evolution of chemically-induced murine hepatomas and to identify the contributions of different genes and gene products to the phenotypes of the transformed cells. Topics under investigation are: (1) identification of phenotypic changes during initiation, promotion and progression; (2) both temporal and cell specific distribution of gene transcripts among normal, preneoplastic and neoplastic liver cells; (3) contribution of liver tumor promoters to gene expression and distribution of transcriptionally active genes.

Methods Employed:

(1) Immunocytochemistry for localization of cell surface receptors using immunoenzyme or immunofluoresence techniques. (2) Radioreceptor assays for quantitation of surface receptors. (3) Cell separation techniques for isolation of preneoplastic cell population. (4) In situ hybridization for spatial localization of m-RNA using autoradiography.

Major Findings:

(1) Transplantation of hepatocytes into the anterior chamber of the eye allows the cells to survive for extended periods that are needed for studying the different stages of hepatocarcinogenesis. (a) Normal hepatocytes attach and proliferate in the anterior chamber. The proliferation is enhanced by the humoral effect of partial hepatectomy. (b) Tumor promoters are toxic for the normal hepatocytes growing in the anterior chamber. However, the growth of preneoplastic and neoplastic hepatocytes is enhanced by tumor promoters. (c) Preneoplastic liver cells may be concentrated by their failure to adhere to tissue culture plates coated with asialofetuin; such cells show a definite growth advantage in the anterior chamber of the rat eye when promoted by phenobarbital. (d) Hepatocytes isolated from animals treated with ultimate carcinogens intraportally a few minutes before the perfusion of the liver and transplanted to the anterior chamber of the eye, survived for several months. However, the thymidine labeling index and the histological staining for gamma-glutamyl transpeptidase did not reveal signs of malignant transformation. In vitro treatment of hepatocytes with ultimate carcinogens gave similar results.

(2) The effect of the liver-specific tumor promoter, phenobarbital on asialoglycoprotein receptor (ASGP-R) was examined in adult rat liver. Both acute and chronic phenobarbital administration decreased the number of receptors per cell determined by radioreceptor assay. Immunofluoresence histochemistry of liver

samples from phenobarbital treated animals revealed centrilobular receptor-deficient areas. In contrast, after partial hepatectomy ASGP-R positive and negative areas were intermingled throughout the liver lobule. Preneoplastic and neoplastic areas displayed uniform reduction in ASGP-R. Four days after birth, the number of hepatocytes with surface receptors was 50% of that in the adult rats. At 10 days after the birth the number of ASGP-R positive cells was the same as in adult rats, although the receptor density was significantly lower than in adults.

(3) Cell differentiation is defined on the basis of cell morphology. In situ hybridization takes into account the morphological aspects of cell differentiation and the changes in the gene expression in these cells. Cytohybridization is a histochemical procedure which combines traditional histology with methods used in molecular biology. It has been used successfully to localize virus derived RNA and DNA molecules. In the earlier methods signal detection was limited to abundant m-RNAs that constitute 1 to 5% of the total poly(A)+RNA population. The development of nick-translated high specific-activity c-DNA has increased the sensitivity of in situ hybridization to include 0.1-0.01% of the total poly(A)+RNA. Thus a high resolution mapping of intracellular m-RNA is now possible. However, depending on the tissue of interest, the conditions for in situ hybridization must be optimized to obtain high signal to noise ratio. Our present goal is to determine (a) appropriate fixation methods for liver samples, (b) appropriate coating methods for slides and cover-slips to obtain sufficient attachment of frozen tissue sections and to prevent non-specific binding of radioactive probes, (c) appropriate proteolytic digestion of frozen sections to allow penetration of the cell membranes by the probes and still maintain the normal morphology of the section, (d).optimal conditions for hybridization (temp. time, etc.), (d) optimal conditions for washing of slides to remove non-specifically bound radioactive probes. The ultimate goal of the technique is to retain the labile m-RNA in the liver cells, and overcome the hindrance of accessibility of radiolabeled probe for cellular m-RNA and simultaneously preserve the intact anatomical structure of liver for subsequent identification of transformed liver cell areas. Our principal goal is to use in situ hybridization to gain insight into the factors that influence the level of specific m-RNA in hepatocytes of intact animals and under the influence of carcinogens and liver tumor promoters. This will be combined with immunohistochemical localization of gene products in the same cells using specific antibodies.

Significance to Biomedical Research and the Program of the Institute.

Our research projects are aimed at increasing the understanding of the multistep process involved in chemical carcinogenesis and thus providing the means to possibly define both cancer cause and to establish effective cancer prevention.

Proposed Course:

Continue the course outlined under Objectives and Major Findings.

Publications:

Evarts, R. P., Marsden, E., Hanna, P., Wirth, P. J. and Thorgeirsson, S. S.: Isolation of preneoplastic rat liver cells by centrifugal elutriation and binding to asialofetuin. Cancer Res. 44: 5718-5724, 1984.

Richards, W. L., Song, M.-K, Krutzsch, H., Evarts, R. P., Marsden, E. and Thorgeirsson, S. S.: Measurement of cell proliferation in microculture using Hoechst 33342 for rapid semiautomated microfluorometric determination of chromatin DNA. Exp. Cell Res. (In Press).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05263-04 LEC

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT (*80 characters or less. Title must fit on one line between the borders.*)

Computer Analysis of Carcinogenesis by Two-Dimensional Gel Electrophoresis

PRINCIPAL INVESTIGATOR (*List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*)

PI:	Mark J. Miller	Senior Staff Fellow	LEC	NCI
Others:	Arthur D. Olson	Computer Programmer	LEC	NCI
	Snorri S. Thorgeirsson	Chief	LEC	NCI
	Gordon L. Hager	Section Head	LEC	NCI
	Peter J. Wirth	Expert	LEC	NCI
	Timothy Benjamin	Chemist	LEC	NCI

COOPERATING UNITS (*if any*)

None

LAB/BRANCH

Laboratory of Experimental Carcinogenesis

SECTION

Chemical Carcinogenesis Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.3	1.2	0.1

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK (*Use standard unreduced type. Do not exceed the space provided.*)

The main objective of this project is to study the mechanism of carcinogenesis using quantitative two-dimensional gel electrophoresis. This technique allows for the simultaneous separation of total cellular polypeptides on a single polyacrylamide gel and allows us to examine qualitative changes in the protein patterns as well as quantitative changes in individual polypeptides as the cell undergoes malignant transformation. Research is, at present, focused on the following areas: (1) continued development and improvements on the computer system (dubbed ELSIE III) currently used to automatically analyze two-dimensional gels; and (2) application of ELSIE III to analyze both the clonal variation among transformed cells and the temporal changes in cellular protein patterns during steroid driven transformation of mouse fibroblasts transfected with MMTV:v-ras chimeras. In the past year we have improved the spot finding program and developed techniques to find spots which vary, either quantitatively or qualitatively, during the course of an experiment. The organization of data on ELSIE III has been modified so that it is now possible to compare gels generated by different experiments. We have examined the polypeptide patterns of 9 single cell clones derived from a clonal cell line, H-4-II-E. Out of 982 proteins found among the various subclones, only 5 qualitative changes in the protein patterns were detected. About 20% of the proteins, however, were synthesized at quantitatively different rates. Finally, we have examined the polypeptide pattern of a clone of NIH3T3 cells, 433. These cells contain the v-ras oncogene and can be induced, by dexamethasone, to synthesize the ras-p21 protein and express the transformed phenotype. Efforts are underway to identify the p21 protein product on these gels and, using ELSIE III, to search for both qualitative and quantitative protein patterns that are associated with the transformation phenotype.

PHS 6040 (Rev. 1/84)

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

Mark J. Miller	Senior Staff Fellow	LEC	NCI
Arthur D. Olson	Computer Programmer	LEC	NCI
Snorri S. Thorgeirsson	Chief	LEC	NCI
Gordon L. Hager	Section Head	LEC	NCI
Peter J. Wirth	Expert	LEC	NCI
Timothy Benjamin	Chemist	LEC	NCI

Objectives:

The main objective of this project is to study the mechanism of chemical carcinogenesis by employing the technique of quantitative two-dimensional gel electrophoresis of total cellular protein. Since this technique allows for the simultaneous separation of total cellular polypeptides on a single polyacrylamide gel, it is possible to follow changes in the rate of synthesis of individual proteins as well as changes in the total protein patterns as the cell undergoes malignant transformation. Our aim is to identify and characterize those proteins that are associated with the transformed phenotype.

Methods Employed:

The principal methods employed are: (1) two-dimensional gel electrophoresis, (2) tissue culture technique, (3) computer-based quantitation of autoradiograms, and (4) radioisotope measurements.

Major Findings:

From its inception, the major objective of the Laboratory's computer facility has been to further expand and develop the two-dimensional gel analysis system in order to facilitate the use of this important research technique in the analysis of the neoplastic process.

A. Advances in the Computerized Analysis of Two-Dimensional Gels. The computer system, developed in this laboratory, for analyzing two dimensional gels has been dubbed ELSIE III. It has been distributed to several other laboratories in the United States and Europe. Some of the more significant advances in the past year are outlined below.

(1) Findspots. A new spot finding algorithm has been developed that combines both the thresholding method described by Vo et al. (Anal. Biochem. 112, 258, 1981) and the negative core detection method of Lemkin (Comp. Biomed. Res. 13, 571, 1981). First, the second derivative of the surface of the gel is calculated in both the X and Y directions using an elliptical least squares convolution template to both smooth the data and calculate the second derivative in one operation. Regions where the second derivative becomes negative, in both directions, identify one or more spots. These "negative cores" regions are next

subjected to a thresholding operation that searches for additional peaks within them. This algorithm improves the accuracy of the spot finding programs without significantly increasing the number of false spots detected (about 1%).

(2) Data Organization. The data for each gel is kept in several files; e.g., one file contains the raw scan data, another the spot shapes, yet another the position and intensity information for each spot. In order to associate each data file with the proper two-dimensional gel and to keep the number of files stored in any directory at a minimum, a single, uniquely named subdirectory is created for each gel and the data files for the gel are stored in that subdirectory. All programs that reference a gel are given only the name of that gel (the subdirectory), the user need not be concerned with the structure and names of the various sub-files that describe the gel. It is the program's responsibility to access the proper files and do the appropriate bookkeeping. This organization also allows us to change the names and structures of data files in a manner invisible to the users of the facility. Finally, the structure allows one to match two gels located anywhere on the computer system simply by specifying the complete path names of the two gel directories. A special file is created that incorporates the pathnames of the gels and the sets of spots matched between the gels.

(3) Analysis Tools. Several program "tools" have been developed to aid in the analysis of processed data. A brief outline of some of the more significant programs follows: (a) Desaturate combines information about heavy and light exposures of a gel. A new set of files is created for the gel in which "saturated" data in the heavy exposure is replaced by the "unsaturated" information in the light. This effectively extends the dynamic range of the gels.

(b) Manypairs matches together unmatched gels among a set of gels where some of them have been matched. For example, if we have four gels, A, B, C and D, where gel A has been matched to gels B, C and D, manypairs can be used to automatically match gel pairs B-C, B-D, and C-D.

(c) Plotcpm plots the intensity of groups of spots as a function of time for gels in an experiment. It can thus be used to plot the kinetics of synthesis of many individual proteins during the course of an experiment.

(d) Findquant finds spots that are statistically different (T test) in intensity between two or more sets of gels.

(e) Findqual uses statistical tests to find spots that are different qualitatively between two or more sets of gels; that is, spots that are intense in at least one set of gels and absent in at least one other.

(4) Measures: picking what to measure to identify unknowns. One way to identify an unknown item is to measure its traits and compare the measurements with those of items the unknown might be. However, it may not be necessary to measure all of the unknown's traits in order to identify the unknown. For example, if a protein were known to be listed in the Protein Sequence Database (PSD -- Georgetown University Medical Center), it is often possible to identify the protein by measuring only some of its 20 amino acids.

Measures is a program which will take known measurements to find the lowest-cost sets of traits to measure which, for learning which of the known times an unknown might be, are as good as measuring all the traits. By labeling cells with different amino acids and using ELSIE III to measure the relative incorporation of label into different polypeptides, it may be possible to simultaneously identify a large number of the proteins resolvable on the gels. The measures program indicates that, depending on the error involved, it should be possible to identify proteins on these gels by labeling with between 12 and 16 different amino acids.

B. An Analysis of Multiple H4-II-E Rat Hepatoma Cell Clones. We were interested in determining the degree of heterogeneity that might appear in subpopulations of "immortal" cell lines. We wished to determine if there were any proteins whose rate of synthesis might vary significantly in independent subclones derived from such a cell line. The rat hepatoma line H4-II-E (Reuber cells) was originally isolated 20 years ago and has been maintained in this laboratory under continuous subculture for several years. Nine independent subclones of H4-II-E were isolated by two rounds of limiting dilution. These are referred to here as the primary subclones. Two of these subclones were resubcloned to isolate several second level subclones (secondary subclones). The cells were maintained under as nearly identical conditions as possible. They were grown in the same incubator, in the same media lots and subclutured at the same time. Labeling, sample preparation, and electrophoresis of each was done simultaneously. Duplicate gels were run of each subclone and of two independent cultures of the primary subclones. The two-dimensional protein patterns were analyzed using ELSIE III.

Qualitative differences between spots in the primary and secondary subclones were very rare. Only 5 spots, out of 982, were found that were consistently present in the primary subclones and absent in the secondary, or vice versa. Of these, three spots appeared to be modification products; that is, another nearby spot of the same molecular weight was reduced in intensity when these spots appeared. Of the two others, one appeared in the primary subclones, but was missing in three of the secondary. The fifth spot was present in all of the secondary subclones, but not detectable in any of the primary. Quantitative differences on the other hand were not uncommon. The findquant program was set to flag polypeptides that were at least two-fold different in relative intensity and where the student's T test indicated the distributions of intensities were different to at least the 99% probability level. Fully 494 of 982 spots matched among the various clones were flagged as being quantitatively different in at least one of the secondary subclones. The difference in intensity of some of these spots in the various clones was as great as 14-fold. On the other hand, many were very faint spots where the accuracy of the quantitation is rather poor. Fully 310 (63%) had an average intensity that fell into the lowest 10% of all spots. Faint but broad spots may contain significant total intensity. Since the spot finding program first looks for spots that have a certain minimum peak intensity, such spots can easily fall below the threshold of detection in different gels and be flagged as being quantitatively different. Forty-four of the 494 spots were chosen at random and more closely examined. Of these, 19 appeared to be consistently different when examined visually; the others were either streaks, or too broad and light to be certain, or focused in a portion of one gel that was missing in other gels (generally at the edges). Hence, approximately 20% of the spots in the secondary subclones were judged to be

quantitatively different from the primary. When findquant compared the duplicate primary subclone cultures, only 20 spots were flagged as being potentially different. None of these were judged to be truly variable when examined visually.

The causes of these differences are unclear. It is unlikely that they are due to mutational events. Likewise, since the differences are not seen in the duplicate primary cultures, most of the differences are not likely to be due to sample preparation artifacts. These relatively small quantitative changes most likely reflect adjustments of the cells to very slight differences in the media or growth environment between the different cell cultures, or the slight differences in degree of confluence of the cells.

C. Analysis of Hormonally Controlled Transformation in NIH3T3 Cells. A clone of NIH3T3 cells transfected with molecular chimeras of Harvey ras oncogene linked to the long terminal repeat (LTR) of mouse mammary tumor virus (MMTV) was isolated by Huang et al. (Cell 27, 245, 1981). This clone, designated 433, expresses normal v-ras gene product, p21, in the presence of dexamethasone. It also reversibly takes on the transformed phenotype when exposed to this drug. We have examined the protein patterns of these cells in the first three days after exposure to dexamethasone in an effort to find early protein changes associated with transformation. During this time significant morphological changes take place in the 433 cells: they become spindle-like, clump together and detach from the culture dish. The parental, NIH3T3 cells appear unaffected. A number of quantitative differences in the rate of polypeptide expression have been observed between the untreated parental NIH3T3 and the 433 cells. However, the two-dimensional gel patterns of the dexamethasone treated and untreated cells are very similar in both cell types. So far, only one set of high molecular weight proteins has been observed to decrease in the presence of dexamethasone in the 433 cells while remaining steady in the NIH3T3s. Detailed quantitative analysis of this experiment, using ELSIE III, is currently underway. Efforts are also being made to identify the p21 protein product on these gels and to search for both qualitative and quantitative protein patterns that are associated with the transformation phenotype using a variety of electrophoretic conditions.

Significance to Biomedical Research and the Program of the Institute:

The technique of two-dimensional gel electrophoresis provides a virtual "snapshot" of the metabolic activity of a cell under a specific set of environmental conditions. Our laboratory's computer system gives us the capability of analyzing and cataloging the synthesis of any protein resolved by such gels. We are involved in studies which will analyze, catalog, and compare the capacity of a cell to synthesize its various proteins during both normal development and chemical transformation and to identify and characterize those proteins that are highly associated with the malignant phenotype. These studies should provide clues as to the biochemical nature of the malignant process and provide a means to identify the number of genes involved in this process.

Proposed Course:

Continue the course outlined under Objectives and Major Findings.

Publications:

Green, M. R., Hatfield, D. L., Miller, M. J. and Peacock, A.: Prolactin homogeneously induces the tRNA population of mouse mammary explants. Biochem. Biophys. Res. Commun. 129: 233-239, 1985.

Miller, M. J. and Olson, A. D.: Automatic analysis of two-dimensional gel electrophoretograms: The processing of multiple gels. In Neuhoff, V. (Ed.): Electrophoresis'84. Weinheim, Germany, Verlag Chemie, 1984, pp. 226-234.

Miller, M. J., Olson, A. D. and Thorgeirsson, S. S.: Computer analysis of two-dimensional gels: Automatic matching. Electrophoresis 5: 297-303, 1984.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01CP05283-03 LEC

PERIOD COVERED
October 1, 1984 to September 30, 1985

TITLE OF PROJECT (*80 characters or less. Title must fit on one line between the borders.*)
Transmission of Mammalian Genes with Expression-Regulated Retrovirus Vectors

PRINCIPAL INVESTIGATOR (*List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Michael G. Cordingley	Visiting Fellow	LEC NCI
Others:	Diana S. Berard	Microbiologist	LEC NCI
	Gordon L. Hager	Head, Hormone Action & Oncogenesis Section	LEC NCI

COOPERATING UNITS (*if any*)

None

LAB/BRANCH
Laboratory of Experimental Carcinogenesis

SECTION
Hormone Action and Oncogenesis Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.2	0.9	0.3

CHECK APPROPRIATE BOX(ES)
☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (*Use standard unreduced type. Do not exceed the space provided.*)

Retrovirus-based vectors are ideally suited for the introduction of genetic information into mammalian cells, both in culture and in vivo. If expression of genes introduced with these vectors could be made conditional, their usefulness would be considerably enhanced. Regulation of expression from a glucocorticoid-induced promoter would be an attractive candidate, since most cell types contain functional glucocorticoid receptors. We have shown previously that expression of the v-Ha-ras oncogene can be made conditional when driven from the glucocortocoid responsive mouse mammary tumor virus (MMTV) promoter, and that the transformed state of cells transfected with these fusions also is dependent on the presence of hormone. A preliminary series of vectors have been constructed with the MMTV V-Ha-ras cassette embedded in a retroviral backbone based on replication competent murine leukemia virus, both in the parallel and anti-parallel transcriptional orientation. The neomycin resistance gene driven from the MuLV promoter has been included to provide selection independent of ras transformation. We find that hormone-dependent expression of the ras oncogene is observed with both orientations of the MMTV v-Ha-ras cassette. These experiments show, in principle, that a retrovirus-based vector can be developed which permits introduction of a given gene into cells with selection pressure independent of the gene, and with subsequent expression of the gene subject to glucocorticoid regulation.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

Michael G. Cordingley	Visiting Fellow	LEC	NCI
Diana S. Berard	Microbiologist	LEC	NCI
Gordon L. Hager	Head, Hormone Action & Oncogenesis Section	LEC	NCI

Objectives:

Construction and characterization of retrovirus-based vectors that permit the efficient introduction of genes into animal cells, both in culture and in vivo, in configurations such that expression of the genes are subject to regulation by glucocorticoid hormone.

The introduction of the ras oncogene, and other candidate oncogenes, into a variety of cell types with these vectors, and subsequent characterization of oncogene activities based upon conditional expression of the oncogene product.

Methods Employed:

Functional elements involved in the replication, transmission and integration of retrovirus based vectors are molecularly cloned using plasmid and bacteriophage vectors.

Ecotropic and amphotropic classes of the MuLV family of murine retroviruses are utilized as the transmission base for construction of vectors.

The v-Ha-ras oncogene is utilized as a test gene for functional expression of oncogenic activity in mouse cells after vector transmission. The neomycin resistance gene is utilized for dominant selection of vector transmission.

Functional expression of the ras gene product is monitored by morphological transformation of susceptible cells. Quantitative levels of ras expression are monitored by immunoprecipitation with monoclonal antibody of ras gene product, or by S1 nuclease analysis of ras transcripts.

Vector DNA intermediates and integrated structures are characterized by restriction endonuclease analysis and Southern transfer blotting.

Vector RNA genomes and transcripts are characterized by S1 nuclease mapping and northern transfer blotting.

Major Findings:

A series of retroviral transmission vectors have been constructed which utilize the LTRs and associated replication functions from MuLV for transmission of the vector, and make use of the glucocorticoid-regulated promoter from MMTV for regulated expression. Transcriptional enhancer elements from a variety of sources are also incorporated as accessory elements to modulate the strength of the MMTV promoter.

Two questions have been addressed. First, functional expression of a retroviral convected gene cassette has been separated from transmission by encoding the information to be expressed on the opposite strand, in the antiparallel sense, and providing it with a promoter separate from the transmission LTRs. Thus functional cassettes that include polyadenylation signals and splicing signals can be transmitted without the problems associated with RNA processing that occurs at these signals when they are included on the transmission strand.

Secondly, we have shown that the ras oncogene can be transmitted into cells with these vectors under conditions where its expression is conditional. That is, when the oncogene is driven from the MMTV promoter in either the parallel or antiparallel configuration, transformation of the cell is dependent on the presence of glucocorticoids.

Significance to Biomedical Research and the Program of the Institute:

Retroviral based vectors have emerged as a powerful tool for the mobilization of genetic information in mammalian cells. When established in a transmissable, defective genome, a given sequence can be introduced into almost any cell type with high efficiency and in a predicted structure. These features are of obvious utility for the manipulation of sequences with oncogenic potential in a variety of cell systems. Furthermore, as an infectious particle, the information can be introduced into the animal, a major advantage for monitoring neoplastic potential. The development of these reagents will be of considerable value in the study of genes implicated in neoplasia, as well as in the mobilization of elements involved in biological regulatory processes.

Proposed Course:

Further development of the vector series will be pursued, with emphasis on the generation of vectors that permit the targetting of information to specific tissues, and regulation of expression once resident in the tissue.

Publications:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05313-03 LEC

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Early Events in Chemically Induced Rat Hepatocarcinogenesis

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	Peter J. Wirth	Expert	LEC	NCI
Others:	Ritva P. Evarts	Veterinary Medical Officer	LEC	NCI
	Timothy Benjamin	Chemist	LEC	NCI
	Brian Huber	Staff Fellow (PRAT)	LEC	NCI
	Dolores M. Schwartz	Biologist	LEC	NCI
	Snorri S. Thorgeirsson	Chief	LEC	NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Experimental Carcinogenesis

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.0	0.9	1.1

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The project was initiated to study the sequence of events during chemically induced neoplasia using the rodent hepatoma model in combination with quantitative two-dimensional electrophoresis (2-D). Results obtained to date include: 1) demonstration that 2-D could reliably detect and quantitate changes in known polypeptide markers for hepatocarcinogenesis (albumin, alpha-fetoprotein, aldehyde dehydrogenase, the individual subunits [Yc, Yb, Ya, and Yp] of the glutathione-S-transferases [B, A, ligandin and P] and DT-diaphorase). 2) Using the Solt-Farber initiation promotion protocol, preneoplastic and neoplastic liver nodules were induced in male Fischer F344 rats. Individual nodules were dissected and classified histologically as being either early preneoplastic, preneoplastic, or neoplastic. One cytosolic polypeptide (pI 6.8/57 kDa) and three membrane-associated polypeptides (pI 6.25/41 kDa; 6.75/26 kDa; 6.05/21 kDa) were expressed in both preneoplastic and neoplastic nodules but not in control liver. In addition to quantitation of the above known markers, numerous quantitative changes were also detected in as yet unidentified polypeptides among the various cell types. Twenty-one membrane and 10 cytosolic polypeptides were down-regulated, while 14 membrane and 6 cytosolic polypeptides were up-regulated during hepatocarcinogenesis. In all but three polypeptides, the direction and magnitude of change were the same in both preneoplastic and neoplastic nodules. 3) Investigation of the homogeneity and heterogeneity of polypeptide expression among individual nodules isolated from separate animals have revealed marked similarities among the individual preneoplastic and neoplastic nodules from the same animal and among nodules isolated from different animals.

PHS 6040 (Rev. 1/84)

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

Peter J. Wirth	Expert	LEC	NCI
Ritva P. Evarts	Veterinary Medical Officer	LEC	NCI
Timothy Benjamin	Chemist	LEC	NCI
Brian Huber	Staff Fellow (PRAT)	LEC	NCI
Dolores M. Schwartz	Biologist	LEC	NCI
Snorri S. Thorgeirsson	Chief	LEC	NCI

Objectives:

The process of chemical carcinogenesis has traditionally been divided into three phases: initiation, promotion, and progression. Although the mouse skin was the first experimental model used to delineate these phases, the rodent liver is another tissue in which the initiation and promotion stages of chemical carcinogenesis can be clearly observed in vivo. The initiation events are generally believed to be the covalent interaction of a carcinogen with critical cellular macromolecules (e.g., DNA, RNA, and proteins) in the target tissues which results in alteration of the normal functions within the cell. These alterations may result in changes in both gene expression and phenotypic characteristics and under appropriate conditions, namely following promotion, develop into malignant tumor cells. Little is known concerning either the early cellular events in the initiation process or the gene products that control and maintain the initiated cells or how these products may change as a function of time during tumor progression. Therefore the main objectives of this project are to characterize the early biochemical events and subsequent changes in gene expression which occur during hepatocarcinogenesis. Using the Solt-Farber initiation-promotion hepatocarcinogenesis protocol, initial studies have focused on the isolation and characterization (histologically, histochemically, and biochemically) of early preneoplastic, preneoplastic, and neoplastic hepatocyte populations and examining differences in polypeptide expression in these populations using quantitative two-dimensional gel electrophoresis of total cellular proteins.

Methods Employed:

The principal methods employed are: (1) tissue culture techniques; (2) cell separation techniques-elutriation centrifugation; (3) histochemical staining; (4) enzyme assays involving radiometric (tritium, carbon-14, sulfur-35, phosphorus-32, and iodine-125) assays; (5) chemical and radiochemical synthesis; (6) differential centrifugation and chromatographic techniques; (7) autoradiography and fluorography; (8) two-dimensional gel electrophoresis; and (9) computer assisted quantitation of autoradiograms and silver stained gels.

Major Findings:

1) Previous biochemical and histochemical studies have demonstrated that the levels or enzymic activities of numerous cytosolic proteins, such as alpha-fetoprotein (AFP), albumin (Alb), DT-diaphorase (DT), aldehyde-NAD(P) oxidoreductase

(aldehyde reductase; AlDH) and various glutathione-S-transferases (GST) as well as the membrane-associated proteins, gamma-glutamyl transpeptidase (GGT), epoxide hydrolase, and the cytochrome P-450 monooxygenases show marked alterations during hepatocarcinogenesis. Initial work was therefore directed towards establishing that 2-D electrophoresis could be used to reliably detect and quantitate changes in these known polypeptide markers during hepatocarcinogenesis.

Albumin (Alb) and AlDH were readily separated on 2D gels using standard O'Farrell conditions (equilibrium isoelectric focusing in the first dimension 13,000 v-hrs). Identification of Alb and AlDH was verified by comigration with highly purified protein preparations and immunoblot analysis (Western transfers) with rabbit anti-rat antibody preparations to Alb and HTC hepatoma purified AlDH (supplied by Dr. Ron Lindahl, Department of Biology, University of Alabama). Previous work has shown that albumin levels are markedly reduced in hepatocellular carcinomas; however, no significant differences in the expression of Alb (pI 6.6/66 kDa) were observed in cytosolic preparations from untreated control liver (0.89 + 0.12% of total integrated density of gel), preneoplastic (1.38 + 0.26%), or neoplastic (1.47 + 0.12) nodules. Although purified hepatoma derived AlDH separates as a single homogeneous band (54 kDa) in 1D (SDS-PAGE) electrophoresis, 2-D electrophoresis separates AlDH as a group of 5 polypeptides with pI values of 6.8-7.1. No differences in the expression of AlDH were observed between preneoplastic nodules and neoplastic nodules; however, polypeptide (b) (pI 6.9), whose constitutive levels in untreated control cells is very low, appears to be shifted slightly toward the basic region in untreated control gels as compared to both preneoplastic and neoplastic nodules. Although AFP has been shown to be markedly increased in hepatocellular carcinomas with concomitant decreases in the expression of Alb, immunoblot analysis with rabbit anti-rat AFP antibody failed to show any AFP production in either preneoplastic or neoplastic nodules.

Initial attempts to identify either DT-diaphorase (DT) or the individual subunits of glutathione-S-transferase (GST) using the standard O'Farrell conditions were not successful due to the basic nature of the subunit polypeptides. DT and the individual Yc (28.5 kDa), Yb (27.5 kDa), and Ya (26 kDa) subunits of GST-A (YbYb), GST-B (YaYc), and ligandin (YaYa), as well as the recently described Yp (26 kDa) subunit of the placental form of GST (GST-P), were, however, separated on 2-D gels under non-equilibrium isoelectric focusing conditions (6000 v-hrs). DT and the subunits of GST were identified by comigration with highly purified enzyme preparations, immunoblot analysis, and comparison with published 2-D electrophoretic patterns of GST-A, GST-B, and GST-P. Marked increases in the expression of the Ya subunit (2-fold) of GST-B (and/or ligandin), the Yb subunit (4-fold) of GST-B and the three isoelectric point variants (Yp) (15-fold) of GST-P were observed in both preneoplastic and neoplastic nodules as compared to untreated control liver. No significant changes in the expression of the Yc subunit of GST-B was observed. The expression of DT-diaphorase (32 kDa) was also increased (2-3 fold) in both preneoplastic and neoplastic nodules as compared to control liver.

Attempts to localize the membrane associated GGT, one of the most reliable markers for preneoplastic liver transformation, from crude membrane preparations from preneoplastic and neoplastic nodules on 2-D gels using immunoblot analysis with antibody preparations to rat kidney GGT, were not successful. Two-dimensional electrophoresis of purified rat kidney GGT showed that GGT is composed of two subunit chains consisting of at least 18 individual subunit polypeptides. Seven

polypeptides (pI 7.0-5.4/23-26 kDa) comprise the light chain and 11 polypeptides (pI 7.1-5.8/51-53 kDa) comprise the heavy chain. Western blot analysis showed that all of these components were immunoreactive with a mixture of the two antibodies raised separately against the light and heavy subunit chains (gifts from Dr. Suresh Tate, Department of Biochemistry, Cornell University). Comparison of 2-D patterns from partially purified GGT from hyperplastic nodules revealed almost identical patterns to the 2-D patterns from purified rat kidney GGT. GGT purified from hyperplastic liver nodules was immunoreactive with antibody (both light and heavy chain) prepared against kidney GGT.

Work is currently in progress toward the localization of epoxide hydrolase and the various cytochrome P-450 isozymes. Due to their basic nature and high lipophilicity it will be necessary to use non-equilibrium isoelectric focusing in the first dimension. Resolution of the various cytochrome P-450 isozymes will be accomplished using immunoblot analysis with various antibody preparations to individually purified cytochrome P-450 species.

2) During the course of hepatocarcinogenesis one of the most important processes in the neoplastic transformation of the liver is the development of focal lesions of proliferative hepatocytes shortly after initiation. After further carcinogen treatment or after promotion, these lesions enlarge to form grossly visible hyperplastic nodules. These nodules have two options: the majority (90-98%) "redifferentiate" back to normal appearing liver while a few persist, enlarge further, and may serve as sites for the formation of the ultimate hepatocellular carcinomas. Since the hyperplastic nodule serves as a critical point in the formation of cancer we have recently begun a study concerning the biochemical nature of these nodules. Male Fischer rats were subjected to the standard Solt-Farber procedure for the induction of the formation of liver hyperplastic nodules. Individual nodules of approximately the same size (3-5 mm) were removed and a small section of each nodule was taken for histological staining (hematoxylin and eosin, GGT, glucose-6-phosphatase). Nodules were classified as being either preneoplastic or neoplastic on the basis of histological examination. All nodules, both preneoplastic and neoplastic, stained strongly for GGT activity. Two-dimensional electrophoretic separation of silver stained polypeptides from normal untreated rat liver tissue and from a neoplastic hyperplastic nodule were very similar although numerous qualitative and quantitiative polypeptide differences were readily detected. Approximately 1100-1200 polypeptides were readily visible on each electrophoretogram. To aid in analysis tissue samples (untreated control liver, preneoplastic, and neoplastic nodules) were fractionated into cytosolic and crude membrane preparations prior to 2-D analysis. Approximately 1000-1100 membrane and 800-1000 cytosolic polypeptides were readily resolved on each electrophoretogram. During hepatocarcinogenesis one cytosolic (A, pI 6.8/57 kDa) and three membrane-associated polypeptides (B, 6.25/41 kDa; C, 6.75/26 kDa; D, 6.05/21 kDa) were expressed in both preneoplastic and neoplastic nodules but not in untreated control liver samples. Quantitatively, polypeptides A, B, C, and D represented 0.11-0.12%, 0.16-0.14%, 0.25-0.28%, and 0.07-0.08%, respectively, of the total integrated density of the respective preneoplastic and neoplastic nodules. For comparison, actin, one of the major membrane associated, and albumin, one of the major cytosolic polypeptides were expressed at concentrations of 1% and 1.5%, respectively. No qualitative spot differences (either in cytosolic or membrane fractions) were observed among preneoplastic nodules and/or neoplastic nodules themselves. Although only four qualitative polypeptide differences were

observed during hepatocarcinogenesis, numerous quantitative polypeptide differences were also detected in both preneoplastic and neoplastic nodules. Quantitative comparisons of polypeptides within the same type of tissues (e.g., untreated vs untreated; neoplastic vs neoplastic; or preneoplastic vs preneoplastic) revealed a relatively tight quantitative correspondence between paired spots in each of the same tissue types in both cytosolic and membrane fractions with correlation coefficients of 0.7-0.9. Greater scattering (correlation coefficients 0.5-0.6) of polypeptide densities were observed, however, when normal untreated liver samples were compared to either preneoplastic or neoplastic nodules. Comparison of polypeptides from two untreated control liver samples revealed that only 1% of the membrane (975 polypeptides compared) and 2.7% of the cytosolic (800 compared) showed quantitative variation greater than four-fold. Examination of the location of the polypeptides showing significant differences showed that many of these were located in regions of the electrophoretograms which are routinely highly variable (i.e. pH > 7.3 MW > 100 kDa). Comparison of 750-1000 membrane and 500-800 cytosolic polypeptides from preneoplastic and neoplastic nodules revealed that roughly 4-8% of the membrane and 6-10% of the cytosolic polypeptides were undergoing quantitative changes of at least four-fold during hepatocarcinogenesis. Polypeptides which showed significant modulation occurred at all pH and molecular weight regions. Twenty-one membrane-associated and 10 cytosolic polypeptides were down-regulated, while 14 membrane and 6 cytosolic polypeptides were up-regulated during hepatocarcinogenesis. In all but three polypeptides, the direction and magnitude of change were the same in both preneoplastic and neoplastic nodules.

3) One of the main characteristics of cancer cells is their marked heterogenicity with respect to cellular structure, biochemistry, immunology, etc., and one hypothesis states that this heterogenicity appears early in the carcinogenic process, possibly during initiation. Others feel, however, that this heterogenicity occurs only late in the process. In an attempt to address this problem, we have begun to investigate the heterogenicity/homogenicity of polypeptide expression in preneoplastic and neoplastic nodules isolated from different animals undergoing hepatocarcinogenesis. Hyperplastic nodules were induced in male Fischer rats (Solt-Farber technique) and 6 hours prior to sacrifice and isolation of hyperplastic nodules animals were treated (i.v. tail vein) with [^{35}S]-methionine (1 mCi). Untreated control animals were treated the same. Nodules were dissected, sections histologically scored as either preneoplastic or neoplastic, and the 2-D electrophoretic analysis of the cytosolic and crude membrane polypeptides performed. At least 20 nodules were analyzed from each animal (3 rats). No significant differences in the incorporation of [^{35}S]-methionine into either membrane-associated or cytosolic polypeptides were observed between preneoplastic and neoplastic nodules and untreated control rat liver. Electrophoretograms were both silver stained to analyze for differences in constitutive polypeptide levels and then subjected to fluorography to analyze for differences in turnover rates of the individual polypeptides. Preliminary data obtained from the analysis of silver stained gels revealed marked homogenicity among the individual nodules isolated from separate animals (3 animals). We are currently analyzing the [^{35}S] labeled polypeptides for differences in turnover rates for the individual polypeptides among the various nodules.

4) Work has been initiated toward the construction of a "liver-polypeptide" map. We have begun a systematic study dealing with the subcellular isolation of the various liver fractions (e.g., nuclei, mitochondria, plasma membranes, nuclear membranes, cytosolic proteins, gap proteins, etc.) and the 2-D electrophoretic analysis of their constitutive polypeptides. Once constructed this map will allow one to focus more closely on those polypeptides which are critically involved in the carcinogenic process(es).

Significance to Biomedical Research and the Program of the Institute:

The liver offers a very attractive model for the study of carcinogenesis since the process of hepatocarcinogenesis appears to follow a "programmed" series of distinctive cellular and tissue changes which ultimately result in the formation of hepatocellular carcinomas. Additional advantages include: broad responsiveness to both initiating and promoting agents; relative homogeneity of cell types within the organ; relative ease of identification and separation of early preneoplastic and neoplastic cell populations. Combined with our laboratory's computer system for the analysis of two-dimensional electrophoretograms of cellular proteins from normal and transformed cells, our studies should provide valuable information as to the biochemical mechanism(s) of chemical carcinogenesis.

Proposed Course:

Continue as outlined under Objectives and Findings.

Publications:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05315-03 LEC

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Cell Surface Proteins and Cellular Adhesion in Hepatocarcinogenesis

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	Hannu Raunio	Visiting Fellow	LEC	NCI
Others:	Snorri S. Thorgeirsson	Chief	LEC	NCI
	Ritva P. Evarts	Veterinary Medical Officer	LEC	NCI
	Ryuichi Konno	Visiting Fellow	LEC	NCI
	Peter J. Wirth	Expert	LEC	NCI
	Joseph DeLarco	Research Chemist	LC	NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Experimental Carcinogenesis

SECTION

Chemical Carcinogenesis Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.5	1.5	0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The main objective of this project is to analyze changes in homotypic cell to cell adhesion during the evolution of chemically induced rat hepatocarcinogenesis, and to identify the cell surface proteins that are involved in this process. The research is currently focused on (1) method developments involving both cell to cell adhesion assays and combination of lecithin based affinity chromatography in combination with two-dimensional gel electrophoresis of cell surface proteins, (2) studies on cell to cell adhesion in normal, preneoplastic and neoplastic hepatocytes, and (3) modulation of transforming growth factors on cell to cell interaction of their target cells. The results so far obtained include: (1) Hepatic cells obtained by perfusing livers with collagenase at different stages of chemically induced (Solt-Farber and/or Peraino method) hepatocarcinogenesis exhibit differential adhesive properties: cells obtained at the preneoplastic nodule stage are more adhesive and cells obtained from a neoplastic stage are less adhesive than normal liver cells. (2) Transforming growth factor beta TGF), when added to the growth medium of normal rat kidney (NRK) cells, elicits a reduction in the ability of the cells to adhere to each other. Epidermal growth factor (EGF) has the apparent capability of reversing the adhesion-impaired effect of TGF .
(3) Clones derived from a Fischer rat liver-derived cell line show markedly varying intracellular adhesive characteristics. The differences in adhesiveness roughly correlates with the chromosome number in the cells in each clone, so that cells that have acquired an aneuploid modal chromosome number tend to be more adhesive to each other than cells that have remained diploid during the cloning process. Two-dimensional gel electrophoresis analysis of concanavalin A binding proteins in the cells showed that the cells from the "most adhesive clone" had quantitatively more protein than the "least adhesive" clone; qualitative changes were little. Neither the clones nor the parent cell line, up to passage 34, were able to grow on soft agar in the presence or absence of EGF.

PHS 6040 (Rev. 1/84)

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

Hannu Raunio	Visiting Fellow	LEC	NCI
Snorri S. Thorgeirsson	Chief	LEC	NCI
Ritva P. Evarts	Veterinary Medical Officer	LEC	NCI
Ryuichi Konno	Visiting Fellow	LEC	NCI
Peter J. Wirth	Expert	LEC	NCI
Joseph DeLarco	Research Chemist	LC	NCI

Objectives:

The main objectives of this project are: (1) to analyze changes in homotypic cell to cell adhesion during different phases of chemically induced rat hepatocarcinogenesis, identify and characterize, primarily by two-dimensional gel electrophoresis, the cell surface proteins that are involved in the cell to cell adhesion, and compare these proteins in normal, preneoplastic and neoplastic cells. Our aim is to identify alterations in cell surface protein patterns that distinguish the malignant cell from the normal cell and to examine the regulation of those surface proteins that are highly associated with the neoplastic process. (2) To characterize the changes in cell to cell adhesion caused by transforming growth factors on their target cells.

Methods Employed:

(1) In vitro culturing of liver-derived epithelial cells and rat kidney cells.
(2) In situ perfusion technique to obtain viable hepatocytes from rat liver.
(3) Cell to cell adhesion assay using an electronic particle counter.
(4) Chromosome analysis using the conventional colcemid-trypsin treatment.
(5) The soft agarose method to delineate anchorage-independent growth of cells.
(6) Analysis of cellular glycoproteins by first selecting the proteins by lecithin affinity chromatography and then applying two-dimensional gel electrophoresis in separating the proteins. (7) Isolation of plasma membrane fractions of livers by using a series of isopycnic and differential centrifugations and a one-step sucrose gradient centrifugation.

Major Findings:

The results so far obtained include: (1) Hepatic cells obtained by perfusing livers with collagenase at different stages of chemically induced (Solt-Farber and/or Peraino method) hepatocarcinogenesis exhibit differential adhesive properties: cells obtained at the preneoplastic nodule stage are more adhesive and cells obtained from a neoplastic stage are less adhesive than normal liver cells. (2) Transforming growth factor beta (TGFβ), when added to the growth medium of normal rat kidney (NRK) cells, elicits a reduction in the ability of the cells to adhere to each other. Epidermal growth factor (EGF) has the apparent capability of reversing the adhesion-impairing effect of TGFβ. (3) Clones derived from a Fischer rat liver-derived cell line show markedly varying intercellular adhesive characteristics. The differences in adhesiveness roughly correlates with the

chromosome number in the cells in each clone, so that cells that have acquired an aneuploid modal chromosome number tend to be more adhesive to each other than cells that have remained diploid during the cloning process. Two-dimensional gel electrophoresis analysis of concavalin A binding proteins in the cells showed that the cells from the "most adhesive clone" had quantitatively more protein than the "least adhesive" clone; qualitative changes were little. Neither the clones nor the parent cell line, up to passage 34, were able to grow on soft agar in the presence or absence of EGF.

Significance to Biomedical Research and the Program of the Institute:

The mechanism(s) by which cells adhere both to each other and to extracellular matrix are, in all likelihood, of fundamental importance to tissue development and differentiation. Therefore, changes in or disruption of these cell to cell interactions that occur during cancer development may be among the crucial factors leading to uninhibited growth and metastasis of cancer cells. Cell surface glycoproteins apparently mediate some of these important cellular events. Basic understanding of both the processes governing the cell-cell and cell-substrate interactions, as well as characterizing the cell surface macromolecules that participate and regulate these processes, may lead to new insight into etiology of human cancers and possibly provide a basis for strategies designed to treat cancer.

Proposed Course:

Continued as outlined under Objectives and Major Findings.

Publications:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05370-02 LEC

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT (*80 characters or less. Title must fit on one line between the borders.*)

Normal Stem Cell Biology and Hemopoietic Regulation

PRINCIPAL INVESTIGATOR (*List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	W. David Hankins	Expert	LEC	NCI
Others:	Peter Klinken	Visiting Fellow	LEC	NCI
	Rou-Lan Qian	Visiting Associate	LEC	NCI
	Kay Chin	Medical Technologist	LEC	NCI
	Nancy Sanderson	Chemist		

COOPERATING UNITS (*if any*)

University of California, Berkeley, CA IJ. Schooley)

LAB/BRANCH

Laboratory of Experimental Carcinogenesis

SECTION

Cell Biology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.5	1.5	1.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK (*Use standard unreduced type. Do not exceed the space provided.*)

Previous studies from this group have documented the secretion of erythropoietin by several isolates of erythroleukemia lines. A relatively rapid and simple purification scheme has been developed for erythropoietin and involved lectin chromatography, gel filtration, ion exchange chromatography, hydroxylapatite chromatography, and finally high pressure liquid chromatography. Polyacrylamide analysis indicates that this material is pure, but sequencing, now in progress, will prove or disprove its homogeneity. This will permit us a route to clone the erythropoietin gene which will open many doors in hemopoietic physiology. In following up on this discovery, we made a provocative observation which has far-reaching implication for cancer detection, therapy and, above all, prevention. We observed that leukemogenic tumor cells could be detected in specially designed hormone sensitivity assays long before leukemia was clinically apparent. The cells which grew in the presence (but not in the absence) of the hormone were shown to produce a fatal leukemia when inoculated back into animals. This sensitivity of the tumorigenic cells led us to test whether reduction of the hormone in vivo might prevent the leukemia from developing. In the majority of treated mice, the tumors disappeared and the animals have appeared healthy for more than one year. If treatment was given later in the disease, the animals did not complete remission but did survive 2-3 times longer than non-treated controls.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

W. David Hankins	Expert	LEC	NCI
Peter Klinken	Visiting Fellow	LEC	NCI
Rou-Lan Qian	Visiting Associate	LEC	NCI
Kay Chin	Medical Technologist	LEC	NCI
Nancy Sanderson	Chemist	LEC	NCI

Objectives:

The research is focused on the following areas: (1) employ current stem cell assays to identify the growth regulators which act upon these primitive cells; (2) continue to devise novel methods for both maintaining the stem cells in culture for longer periods of time and to improve the recognition of stem cells as well as further defining their progeny; and (3) purification, preparation of a panel of monoclonal antibodies and molecular cloning of erythropoietin.

Methods Employed:

(1) Methlycellulose cultures, (2) hematology staining methodology, (3) tissue culture of adherent and non-adherent cells, (4) protein purification procedures (PAGE, gel filtration, affinity columns, etc.), and (5) tritiated thymidine uptake erythropoietin assay.

Major Findings:

We have used our previously identified erythroleukemia cell lines as a source of the hormone for the purpose of purifiying erythropoietin to apparent homogeneity. A relatively rapid and simple purification scheme was developed and involved lectin chromatography, gel filtration, ion exchange chromatography, hydroxylapatite chromatography, and finally high pressure liquid chromatography. Polyarylamide analysis indicates that this material is pure, but sequencing, now in progress, will prove or disprove its homogeneity. This will permit us a route to clone the erythropoietin gene which will open many doors in hemopoietic physiology.

In trying to identify new hormone secreting cell lines, we made the startling observation that leukemogenic cells which had been passaged for over one year, were very sensitive to the growth promoting effects of the hormone erythropoietin, which controls normal red blood cell production. This observation, along with our previously proposed model of carcinogenesis, led us to test whether the leukemia could be treated by an anti-hormone approach. It could! When leukemic mice were treated with procedures that lowered endogenous erythropoietin, the animal lived 2-3 times longer than the untreated counterparts. However, two even more exciting findings emanated from follow-up experiments. These were (A) that tumor cells could easily be detected months before fulminant leukemia developed, and (B) lowering erythropoietin after early detection prevented the leukemia from developing in 7 of 10 mice.

Significance to Biomedical Research and the Program of the Institute:

These findings are highly significant since they demonstrate that tumor cells which induce a lethal leukemia in mice retain hormone sensitivity. This hormone sensitivity could be exploited not only as a basis for effective therapy in the terminal phases of the disease but could be used for early tumor detection and prevention of the progression from "pre-leukemia" into clinical leukemia.

Proposed Course:

This project is being terminated due to reorganization of the Cell Biology Section.

Publications:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05371-02 LEC

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT (*80 characters or less. Title must fit on one line between the borders.*)

Analysis of Direct Actions of Transforming Proteins of Oncogenic Viruses

PRINCIPAL INVESTIGATOR (*List other professional personnel below the Principal Investigator.*) (*Name, title, laboratory, and institute affiliation*)

	Name	Title	Lab	Institute
PI:	W. David Hankins	Expert	LEC	NCI
Others:	Peter Klinken	Visiting Fellow	LEC	NCI
	Rou-Lan Qian	Visiting Associate	LEC	NCI
	Kay Chin	Medical Technologist	LEC	NCI
	Nancy Sanderson	Chemist	LEC	NCI
	Azhar Hossain	Guest Researcher	LEC	NCI

COOPERATING UNITS (*if any*)

Massachusetts Institute of Technology, Cambridge, Ma (R. A. Weinberg and R. Mulligan)

LAB/BRANCH

Laboratory of Experimental Carcinogenesis

SECTION

Cell Biology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.8	1.3	0.5

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK (*Use standard unreduced type. Do not exceed the space provided.*)

We have attempied to develop the methodology which will allow the transfer of cloned genes into hemopoietic stem cells. Hemopoietic stem cells were chosen because (A) these cells are multipotential, (B) can be cultured in vitro permitting analysis of individual progeny, and (C) will reconstitute the hemopoietic compartment of a lethally irradiated mouse. This latter propery makes feasible the study of long-term regulation in vivo and in vitro and also opens new possibilities for genetic therapy of such hemopoietic diseases as sickle cell anemia and thalassemia. We have approached these experiments from two directions. First, we have gained substantial experience with making viral constructs containing genes of interest and appropriate regulatory sequences. We have now completed work which demonstrates that constructions containing the EJ bladder carcinoma gene will infect and transform erythroid progenitors in a manner that provides a growth advantage without blocking differentiation. As the erythroid cells mature and synthesize hemoglobin and other red cell proteins, they also synthesize copious amounts of the oncogene protein p21. Second, we have used similar constructs to demonstrate for the first time that multipotent hemopoietic stem cells can be directly infected and that their differentiated progeny will express the transferred genetic information.

PHS 6040 (Rev. 1/84)

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

W. David Hankins	Expert	LEC	NCI
Peter Klinken	Visiting Fellow	LEC	NCI
Rou-Lan Qian	Visiting Associate	LEC	NCI
Kay Chin	Medical Technologist	LEC	NCI
Nancy Sanderson	Chemist	LEC	NCI
Azhar Hossain	Guest Researcher	LEC	NCI

Objectives:

The long-term goals of this project are to (1) analyze transforming genes in hemopoietic cells, (2) define unknown functions of cellular genes, (3) study globin gene regulation, and (4) examine genetic therapy.

Methods Employed:

(1) Northern, Southern, and Western blotting techniques to identify transforming DNA, RNA or protein, respectively; (2) preparation of retroviral vectors carrying transforming sequences; (3) in vivo and in vitro hemopoietic stem cell assays; (4) gene cloning procedures; (5) in situ hybridization; and (6) in vitro virus-transformation assays.

Major Findings:

We have continued our study of the effects of virus transforming genes on hemopoietic cells. We have now been able to prepare retroviral vectors which carry genes into specific types of hemopoietic cells. This will allow the study of many of the questions associated with tissue specific gene regulation, enhancer elements, etc. In particular, we now can determine the effects of putative regulatory sequences in erythroid precursors as they differentiate. This is possible because of our demonstration that vectors carrying p21 genes become integrated into and are expressed in erythroid precursors after direct infection in vitro.

We have now moved on to ask whether it is possible to infect a single multipotential cell and if so, whether the genes are expressed in the different lineages which are the progeny of that cell. A manuscript is in preparation detailing this work which clearly demonstrates that the viral gene ras can be transferred into a single hemopoietic stem cell. When the cell is subcultured under the appropriate hormonal conditions to allow blood cell differentiation, p21 expression in some, but not all, progeny is unequivocal.

Significance to Biomedical Research and the Program of the Institute:

A great deal of current scientific information indicates that oncogenes are fundamentally involved in carcinogenesis. However, many questions remain as to how these genes function in both the normal cell as well as abnormal cancer cells. These experiments provide a direct route toward answering such questions.

Proposed Course:

This project is being terminated due to reorganization of the Cell Biology Section.

Publications:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01CP05373-02 LEC

PERIOD COVERED
October 1, 1984 to September 30, 1985

TITLE OF PROJECT (*80 characters or less. Title must fit on one line between the borders.*)
Purification of Rat Hepatic Proliferation Inhibitor (HPI)

PRINCIPAL INVESTIGATOR (*List other professional personnel below the Principal Investigator.*) (*Name, title, laboratory, and institute affiliation*)

PI:	Henry C. Krutzsch	Expert	LEC	NCI
Others:	Snorri S. Thorgeirsson	Chief	LEC	NCI
	Ryuichi Konno	Visiting Fellow	LEC	NCI
	James B. McMahon	Expert	LETM	NCI

COOPERATING UNITS (*if any*)
None

LAB/BRANCH
Laboratory of Experimental Carcinogenesis

SECTION
Biopolymer Chemistry Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.0	2.0	0

CHECK APPROPRIATE BOX(ES)
☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (*Use standard unreduced type. Do not exceed the space provided.*)

The aim of this project is to isolate and study the endogenous polypeptide from rat liver that arrests growth of normal hepatocytes in a reversible (cytostatic), rather than irreversible (cytotoxic), fashion. The first objective of this work is to arrive at a purification scheme for isolation of this hepatic proliferation inhibitor (HPI), so that sufficient material is then available for structural and biological studies. Structural studies will allow comparison with known polypeptide factors, antibody production and gene cloning. Biological studies will focus on determination of such things as mechanism of action, detection of receptor, and interaction with growth factor pathways. A preparative scheme now appears to be in hand to allow acquisition of sufficient amounts of HPI for the structural and biological studies mentioned above to be carried out. The purification scheme for this molecule, initially a modification of previous methodology, has evolved into an essentially entirely new procedure. The steps now used in the isolation of HPI from rat liver involve a two step homogenization of tissue, acidification of the homogenate, centrifugation, neutralization of the supernatant, centrifugation, ammonium sulfate precipitation and ethanol precipitation. This is followed gy a series of column chromatographic steps involving phenyl sepharose, gel filtration, cation and anion exchange FPLC, and weak anion exchange HPLC. Approximately a 200,000-fold purification of HPI was achieved from this series of steps. The bioassay for the elution position of HPI during these chromatographies was the one previously developed in this laboratory and involves a simple and rapid method, with the use of 96-well microtiter plates for cell culture, a tritiated thymidine uptake assay using a PhD cell harvester, and an assay for total cell DNA content using a viable fluorescent dye and an automatic microtiter plate fluorescence reader. HPI has an ED 50 in approximately nanomolar concentrations, is stable to acid, DTT reduction, heat (50°C), and is a single polypeptide chain of 25K daltons.

PHS 6040 (Rev. 1/84)

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

Henry C. Krutzsch	Expert	LEC	NCI
Snorri S. Thorgeirsson	Chief	LEC	NCI
Ryuichi Konno	Visiting Fellow	LEC	NCI
James B. McMahon	Expert	LETM	NCI

Objectives:

The objective of this project is to isolate and study the endogenous polypeptide in liver that inhibits hepatocyte proliferation, called hepatic proliferation inhibitor or HPI. The initial goal of the project is to define a route for the purification of sufficient amounts of HPI so that extensive characterizations of this molecule can then be undertaken. Structural studies will allow antibody production, DNA cloning and comparison with other biologically active proteins. Biological studies will focus on such important aspects as mechanism of action, detection of the HPI receptor, mode of action of the receptor, and interactions with growth factor action pathways.

Methods Employed:

For HPI isolation in this work, standard procedures for tissue homogenization, ammonium sulfate and ethanol precipitation, column chromatography and fast protein liquid chromatography (FPLC) conditions were utilized. The assay employed to detect HPI activity during chromatography was the new rapid assay, developed in this laboratory, that uses 96 well microtiter plates and measures the total cellular DNA in each well by fluorescence means and the rate of DNA synthesis by determination of tritiated thymidine uptake in each well.

Major Findings:

The first goal in the effort to purify HPI was to develop a rapid and simple assay that would allow the screening of many fractions at once. With this objective accomplished, a major effort was then focused on defining a route for purification of sufficient amounts of HPI so that the studies described under Objectives could be carried out. This second goal now appears to have been met. Starting with a previous preparative scheme for HPI, a totally new procedure has been developed for isolation of this molecule. The purification now used employs the following steps: 1) homogenization of livers in a Waring blender followed by a second homogenization with a high speed Polytron type of instrument; 2) acidification of the homogenate, centrifugation, neutralization of the supernatant, centrifugation; 3) ammonium sulfate precipitation followed by ethanol precipitation; 4) phenyl sepharose, then gel filtration column chromatography; 5) FPLC cation exchange chromatography followed by FPLC anion exchange chromatography; and 6) weak anion exchange HPLC. One dimensional gel electrophoresis after anion exchange FPLC showed one major band and several very minor bands in addition to a major band at the position where HPI would be, and after weak anion exchange HPLC showed essentially only one band corresponding to the position of HPI.

Some studies were carried out with partially purified HPI. Stability experiments showed HPI to be rather acid stable, with half the activity retained after 2 hours at pH 1.5 at room temperature, and the same result was obtained after treatment with dithiothreotol. HPI was found to be stable after treatment at 50°C for 2 hours but all activity was abolished at 70°C for 2 hours. This molecule was also rather unstable toward organic solvents at low pH, which precluded purification in an active form by reverse phase HPLC. Attempted chromatography on a variety of polysaccharide binding columns indicates HPI probably has no post-translational modification with oligiosaccharide side chains. The results obtained on the one dimensional gel analyses, which were carried out under reducing conditions, shows that HPI probably consists of a single polypeptide chain of about 25,000 daltons in size. All of these results and several anti-TGF beta antibody studies showed that the HPI that has been studied here is a different molecule than TGF beta, another inhibitor of hepatocyte proliferation.

Significance to Biomedical Research and the Program of the Institute:

This research project is aimed at increasing the understanding about polypeptide and protein factors that control or are involved in normal and neoplastic cell proliferation and transformation. This knowledge should provide further insight into the oncogenic process and give clues to its control.

Proposed Course:

Continue as outlined under Objectives and Major Findings.

Publications:

Cheng, K. C., Krutzsch, H. C., Grantham, P., Park, S. S., Gelboin, H., and Friedman, F.: Amino-terminal sequence analysis of six cytochrome P-450 isozymes purified by monoclonal antibody immunoprecipitation. Biochem. Biophys. Res. Commun. 123: 1201-1208, 1984.

Deibler, G. E., Krutzsch, H. C., and Martenson, R. E.: A reexamination of the amino acid sequences of bovine, rabbit, monkey and human myelin basic proteins. J. Biol. Chem. 260: 472-474, 1985.

Deibler, G. E., Martenson, R. E., Krutzsch, H. C., and Kies, M. W.: Sequence of guinea pig myelin basic protein. J. Neurochem. 43: 100-105, 1984.

Fairwell, T., Krutzsch, H. C., Hempel, J., Jeffrey, J., and Jornvall, H.: Acetyl blocked N-terminal structures of sorbitol and aldehyde dehydrogenases. FEBS Lett. 170: 281-289, 1984.

Friedman, F. K., Robinson R. C., Krutzsch, H. C., Grantham P. H., Park, S. S., and Gelboin, H. V.: Monoclonal antibody directed isolation and amino-terminal sequence analysis of phenobarbital induced rat liver cytochrome P-450. Biochem. Biophys. Res. Communun．(In Press)

Gajewski, E., Dizdaroglu, M., Krutzsch, H. C., and Simic, M. G.: OH radical induced cross-links of methionine peptides. Int. J. Radiat. Biol. 46: 47-55, 1984.

Kira, J.-I., Deibler, G. E., Krutzsch, H. C., and Martenson, R. E.: Amino acid sequence of porcine myelin basic protein. J. Neurochem. 44: 133-142, 1985.

Krutzsch, H. C.: Polypeptide sequence analysis using gas chromatography-mass spectroscopy. In Shively, J. E. (Ed.): Microcharacterization of Polypeptides: A Practical Manual. New Jersey, Humana Press. (In Press)

Law, M. J., Deibler, G. E., Martenson, R. E., and Krutzsch, H. C.: Plasmin sensitive bonds in rabbit myelin basic protein. J. Neurochem. (In Press)

Marasco. A., Phan, S. H., Krutzsch, H. C., Showell, H. J., Feltner, D. E., Nairn, R., Becker, E. L., and Ward, P. A.: Purification and identification of formyl-methionyl-leucyl-phenylalanine as the major peptide neutrophil chemotactic factor produced by Escherichia coli. J. Biol. Chem. 259: 5430-5439, 1984.

Richards, W. L., Song, M.-K. H., Krutzsch, H. C., Evarts, R. P., Marsden, E., and Thorgeirsson, S. S.: Measurement of cell proliferation in microculture using Hoechst 33342 for the rapid semiautomated microfluorometric of chromatin DNA. Exp. Cell Res. (In Press)

Song, M.-K. H., Krutzsch, H. C., Hankins, W. D., Richards, W. L., and Thorgeirsson, S. S.: Rapid determination of DNA synthesis in microtiter plates. Exp. Cell Res. 156: 271-273, 1985.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05374-02 LEC

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Structural and Physicochemical Studies of Proteins Relevant to Tumorigenesis

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

	Name	Title	Lab	Institute
PI:	Peter P. Roller	Head, Biopolymer Chemistry Section	LEC	NCI
Others:	Snorri S. Thorgeirsson	Chief	LEC	NCI
	James L. Cone	Chemist	LEC	NCI
	Preston H. Grantham	Chemist	LEC	NCI
	Irene B. Glowinski	Staff Fellow	LEC	NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Experimental Carcinogenesis

SECTION

Biopolymer Chemistry Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.7	2.0	0.7

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

This project involves studies on the chemical structure, molecular conformation, and physicochemical characteristics of certain natural biopolymeric materials and their synthetic analogs with the aim of relating the resulting structural information to their biological mode of action. Current emphasis is focused on proteins that play a role in cell growth regulation, cell transformation or differentiation. Emphasis is placed on applying the modern spectroscopic methodologies of mass spectrometry, nuclear magnetic resonance and circular dichroism, as well as the standard methods of protein sequencing, to the solution of these problems. Projects include: 1) Structural studies on gamma-glutamyl transpeptidase (GGT), a tumor marker enzyme of unknown structure. A rapid HPLC method was developed to preparatively separate the enzyme into its subunits. Two dimensional gel electrophoresis on the active enzyme demonstrated 7 isozymic components in the 24,000 molecular weight range and at least 11 isozymic components in the 52,000 molecular weight range. All electrophoretically separated components were found to be immunoreactive to anti-GGT antibodies. N-Terminal amino acid sequencing was accomplished on the light and heavy subunits to the extent of 36 and 32 residues, respectively. The resulting data allows for further work in the area of gene cloning studies and also in comparison of the enzyme found in various biological matrices. 2) Fast atom bombardment mass spectrometry. Spectral measurements on standard peptides indicates that the method is applicable for obtaining valuable accurate molecular weight information on peptides in the several thousand mass range, complementing but not displacing the results obtainable by classical sequencing techniques. Group specific derivatization by brominated reagents were found to improve our predictive ability to sequence peptides. These methods are also being applied to blocked peptides, such as N-terminal pyroglutamates, where classical sequencing techniques are not effective.

PHS 6040 (Rev. 1/84)

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

Peter P. Roller	Head, Biopolymer Chemistry Section	LEC	NCI
Snorri S. Thorgeirsson	Chief	LEC	NCI
James L. Cone	Chemist	LEC	NCI
Preston H. Grantham	Chemist	LEC	NCI
Irene B. Glowinski	Staff Fellow	LEC	NCI

Objectives:

(1) To study in some detail the chemical structure, molecular conformation and physicochemical characteristics of certain natural biopolymeric materials, with current emphasis on proteins that play a role in cell growth regulation and cell transformation or differentiation, such as enzymes, hormones, growth factors and transforming factors, whose level of expression, and/or molecular structure is aberrantly modified during these biological processes. In particular, to study in detail the accurate chemical structural features and the isozymic composition of the tumor marker protein, gamma-glutamyl transpeptidase. (2) Development and applications of modern fast atom bombardment mass spectrometric methods for protein sequencing and structure determination as a complementary tool to the classical methodologies. (3) To study the secondary and tertiary structure of proteins and other biopolymers and the changes in the conformation of these molecules caused by carcinogens, external agents that bind to them, or by minor modifications in the structure, using modern spectroscopic methods. (4) To relate the physicochemical characteristics of macromolecules to their biological functions.

Methods Employed:

(1) Fast atom bombardment negative and positive ion mass spectrometry; (2) proton and carbon-13 nuclear magnetic resonance spectroscopy on samples in the solution state; (3) circular dichroism spectropolarimetry; (4) spectrophotometry; (5) chemical modification and derivatization of peptides and proteins; (6) high pressure liquid chromatography, gel filtration, partition and affinity chromatographies; (7) two dimensional gel electrophoresis; (8) enzymatic proteolysis of glycoproteins; (9) Edman sequencing of proteins; and (10) amino acid analysis.

Major Findings:

(1) Chemical structure studies on gamma-glutamyl transpeptidase (GGT). gamma-Glutamyl transpeptidase (GGT) is a membrane bound glycoprotein enzyme of unknown structure that is involved in the degradative metabolism of glutathione and the uptake of some amino acids. In rodents GGT activity is especially high in fetal liver and in adult kidney but not in adult liver. The low liver enzyme activity is inducible by certain drugs and carcinogens such as azo dyes. For example, elevated levels of GGT are found in most hepatomas. This enzyme is thus expected to be a marker of neoplastic transformation, especially in hepatoma cells. It is also known that there are structural differences between the enzymes found in tumorigenic tissue and the corresponding normal tissues.

All structural work was carried out on an enzyme isolated from normal adult rat kidneys, so that the data can serve as a basis for comparison of the enzyme from other sources. A 170-fold enzyme purification was achieved providing an electrophoretically pure preparation. It is known from earlier work that the enzyme is a heterodimer. Our selected method of purification involved freeing the enzyme from the short membrane binding hydrophobic segment of the heavy subunit with papain, followed by various chromatographies. A fast semimicro preparative HPLC method was developed to separate the two enzymatic subunits under acidic conditions, but in the absence of detergents, buffers and denaturants, thus providing suitable samples for chemical sequencing. Two dimensional (2-D) gel electrophoresis was found to resolve the active papain purified enzyme into at least 18 components. Seven components with apparent molecular weight of 23-26,000 and isoelectric point range of 5.4-7.0 comprise the light subunit, and 11 components with apparent molecular weight of 51-53,000 and isoelectric point range of 5.8-7.1 comprise the heavy subunit. Immunoblot analysis of 2-D gels showed that all of these components are immunoreactive with a mixture of the two antibodies generated separately against the light and heavy subunits, demonstrating that they are all valid constituents of the enzyme complex. N-Terminal amino acid sequencing of the separated subunits of the papain purified enzyme yielded for the first time sequence information for the first 32 residues of the heavy chain with terminal starting sequence of Gly-Lys-Pro-Asp-His-Val-Tyr-Ser-Arg-Ala, and for the first 36 residues of the light subunit with terminal starting sequence of Thr-Ala-His-Leu-Ser-Val-Val-Ser-Glu-Asp. The noncovalently bound light subunit is surprisingly nonpolar, 19 of its amino acids being hydrophobic, and only 5 being the charged type. Whereas the 2-D gel electrophoresis results indicate considerable heterogeneity for the active enzyme, the sequencing information, at least to the extent determined here, confirms earlier observations that the heterogeneity does not reside in the amino acid sequence of the enzyme, but rather in the attached carbohydrate chains.

(2) Peptide sequencing by fast atom bombardment mass spectrometry. The classical Edman sequencing of proteins and peptides is a time proven technique for structural analysis. There are numerous situations, however, where this technique fails or where the results are ambiguous. In those cases the rapidly developing technique of fast atom bombardment (FAB) mass spectrometry can serve as an invaluable alternative. FAB mass spectrometric measurements allow determination of molecular weights to within 1 mass unit or better on peptides with amino acid residues of 25 and possibly more, a molecular weight range eminently suited, for example, for tryptic peptide analysis. For example, we successfully observed the molecular ion for the 34 residue synthetic fragment of the p21 *ras* protein (prepared by C.-H. Niu of our laboratory). It would be desirable to extract sequence information also from the mass spectra. Unfortunately, this information, especially when obtained on subnanomole amounts of sample, is not satisfactory. In an effort to overcome this limitation we are developing methods with model peptides, using group specific reagents such as those described below, to increase the predictable charge localization in the molecule once it is ionized, and also to simplify the fragmentation pattern in an effort to give more reliable sequence information.

One set of important N-terminally blocked peptides that poses problems with the classical Beckman method of sequenation is the N-terminal pyroglutamates. We have examined the spectra of several synthetic pyroglutamates in the positive

FAB mode of operation. Data indicate that the ionization efficiency of these compounds is lower than it is for underivatized peptides. There is, however, valuable sequence information in the spectra. For example, in the spectra of the nonapeptide, serum thymic factor, charge retention appears on the amino terminal end of the molecule showing informative serial fragments with cleavage of the amide nitrogen and the α-carbon atom of the neighboring amino acid. We are also planning to apply the bromobenzoylation procedure, which we have used more extensively in the past and reported on in last year's reports, for tagging basic sites in these peptides, and thus to be able to locate the sequence position of such sites. Bromine containing tags can also be attached to the carboxy terminal end of peptides by making, for example, the p-bromophenacyl ester derivatives. The bromine atom in these derivatives can act as a marker, since it consists of an even mixture of two isotopes two mass units apart, and thus ions containing the amino terminal segment of the peptide will show up as doublets. Examination of the spectra of a number of underivatized peptides indicates that the low molecular weight region of the spectrum may contain valuable information about the actual amino acid content of a peptide. The principal ion in this connection is an iminium ion of the type $RCHNH_2^+$. Among others phenylalanine, leucine, valine and tyrosine give especially significant ions. The methodology developed in our laboratory is directly applicable to relevant structural problems. We have applied it to date for the structural confirmation of synthetic peptides, prepared in our laboratory, and it should prove to be useful in complementing the classical Edman methodology and the dipeptidase method of GC-MS sequencing approaches.

Significance to Biomedical Research and the Program of the Institute:

Establishment of molecular structure of relevant biomolecules by up-to-date spectroscopic and other methods is a necessary prerequisite in a modern approach for ultimate understanding of the complex molecular transformations taking place in living systems, particularly in cancer causation mechanisms. We are applying existing methodologies to solve the structural and functional roles of biopolymers, particularly of proteins and marker enzymes that are causative or indicative of the neoplastic transformation. The amino acid sequence information on GGT, described above, is sufficient for construction of DNA probes necessary for cloning of the GGT gene. That information will ultimately allow studies on the gene regulation of this marker enzyme. The optimized mass spectral analytical method can be applied for detecting post-translational covalent modification of proteins, for sequencing amino terminal blocked protein fragments and for confirmation or correction of gene sequences coding for relevant proteins.

Proposed Course:

Continue the course outlined under Objectives and Major Findings and expand the application of these methodologies to a wider variety of biological problems especially to ascertain structural aspects that the gene sequencing method may not provide.

Publications:

Fox, C. H., Johnson, F. B., Whiting, J. and Roller, P. P.: Formaldehyde fixation. J. Histochem. Cytochem. (In Press).

Hwang, K., Stelzig, D. A., Barnett, H. L., Roller, P. P., and Kelsey, M. I.: Purification of the growth factor mycotrophein. Mycologia 77: 109-113, 1985.

Kroeger-Koepke, M. B., Michejda, C. J., Roller, P. P., and Keefer, L. K.: Use of 3,4-dichlorobenzenethiol as a trapping agent for alkylating intermediates during in vitro metabolism of nitrosamines. Cancer Res. 45: 2973-2975, 1985.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05375-02 LEC

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Effect of Chemical Leukemogens on Hemopoietic Target Cells

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	Vanessa T. Vu	Staff Fellow	LEC	NCI
Others:	Snorri S. Thorgeirsson	Chief	LEC	NCI
	Peter P. Roller	Head, Biopolymer Chemistry Section	LEC	NCI
	W. David Hankins	Expert	LEC	NCI
	Miriam Falzon	Visiting Fellow	LEC	NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Experimental Carcinogenesis

SECTION

Biopolymer Chemistry Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.4	1.4	0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The objective of this project is to study the mechanism(s) of chemically induced leukemia. Our approach is focused on (1) developing in vivo model(s) in which specific types of leukemia are elicited rapidly and in high yield by chemicals in rodents, (2) utilizing these systems to characterize the alterations of the proliferation and differentiation of hemopoietic cells at different stages of leukemia in order to better define the target(s) of transformation and the sequence of cellular events which mediate the progression of neoplasia, (3) investigating the biological interactions of chemical carcinogens and tumor promoters on hemopoietic target cells in vitro, and (4) characterizing the covalent interactions of chemicals with DNA in hemopoietic tissues to gain further knowledge of the initiation stage of chemically induced leukemia. Chemicals which are under investigation include 7,12-dimethylbenz[a]anthracene (DMBA), 7,8,12-trimethylbenz[a]-anthracene (TMBA), N-methylnitrosourea (MNU), N-hydroxy-2-acetylaminophenanthrene (N-OH-AAP), N-acetoxy-2-acetylaminofluorene (N-OAc-AAF), and 12-O-tetradecanoyl-phorbol-13-acetate (TPA). Results obtained so far are (1) detection of DMBA-DNA adduct formation in bone marrow and spleen cells of rats following an i.v. dose of DMBA, (2) development of cell culture clonal assays detecting an array of rat and mouse hemopoietic progenitor cells, (3) demonstration of a stimulatory effect of TPA on monopotent and bipotent hemopoietic cells but a lack of any effect of DMBA, MNU, N-OH-AAP, and N-OAc-AAF on various progenitors in cell culture. Future studies will include (1) continuation of the development of a method to elicit erythroleukemia in rats by DMBA and TMBA, (2) characterization of the colony formation ability in cell culture of hemopoietic progenitors in DMBA and TMBA treated rodents, and (3) determination of in vivo formation of DNA adducts with TMBA in hemopoietic tissues.

PHS 6040 (Rev. 1/84)

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

Vanessa T. Vu	Staff Fellow	LEC	NCI
Snorri S. Thorgeirsson	Chief	LEC	NCI
Peter P. Roller	Head, Biopolymer Chemistry Section	LEC	NCI
W. David Hankins	Expert	LEC	NCI
Miriam Falzon	Visiting Fellow	LEC	NCI

Objectives:

A number of chemicals have been shown to induce leukemia in human and experimental animals. Previous in vivo studies indicate that certain chemicals elicit only specific types of leukemia (e.g., erythroid, myeloid, lymphoid) depending on species, strain, sex and age of the animals, suggesting that chemical leukemogens may act on different target cells. This project is aimed at elucidating the mechanism(s) of chemically induced leukemia. Our approach is focused on (1) developing in vivo model(s) in which specific types of leukemia are induced rapidly and in high yield by chemicals in rodents, (2) utilizing these systems to characterize the alterations of the proliferation and differentiation of hemopoietic cells at different stages of leukemia in order to better define the target(s) of transformation and the sequence of cellular events which mediate the progression of neoplasia, (3) investigating the biological interactions of chemical carcinogens and tumor promoters on hemopoietic target cells in vitro, and (4) characterizing the covalent interactions of chemical leukemogens with cellular DNA in hemopoietic tissues in vivo to gain further knowledge of the initiation stage of chemically induced leukemia. Chemicals which are under investigation include 7,12-dimethylbenz[a]anthracene (DMBA), 7,8,12-trimethylbenz-[a]anthracene (TMBA), N-methylnitrosourea (MNU), N-hydroxy-2-acetylaminophenanthrene (N-OH-AAP), N-acetoxy-2-acetylaminofluorene (N-OAc-AAF), and 12-O-tetradecanoylphorbol-13-acetate (TPA).

Methods Employed:

(1) HPLC analyses of carcinogen-DNA adducts using radiolabeled substrates, (2) UV and fluorescence spectroscopy for structural confirmation of carcinogen-nucleoside adducts, and (3) hemopoietic stem cell colony assays using bone marrow and spleen cells from normal or treated rats and mice grown in methylcellulose culture in the presence or absence of growth regulators including erythropoietin and colony stimulating factors which are present in pokeweed mitogen stimulated conditioned media or Wehi-3 conditioned media.

Major Findings:

(1) We have initiated the in vivo experiments in which female Long-Evans rats were given four intravenous pulse doses of DMBA and TMBA at biweekly intervals beginning at age 28 days. Hematologic examination is routinely performed on blood drawn by the tail vein under brief ether anesthesia to determine the onset of leukemia. Mammary carcinoma has recently been detected in a number of TMBA treated rats.

(2) We have successfully developed primary cell culture clonal assays detecting both rat and mouse hemopoietic progenitor cells which form pure and mixed colonies consisting of mature and morphologically identifiable cells of more than four lineages, as well as blast cell colonies which are composed of only undifferentiated blast cells. In the secondary cell culture clonal assays, these blast cells can give rise to pure and mixed colonies, many of which consist of cells of three to four lineages.

(3) We have used these assays to study the interactions of chemicals on the proliferation and differentiation of hemopoietic cells in vitro. Treatment of marrow and spleen cultures with chemical leukemogens including DMBA, MNU, N-OH-AAP and the hepatocarcinogen, N-OAc-AAF showed no effect on the colony formation of an array of normal murine hemopoietic progenitor cells, with the exception of nonspecific cytotoxicity at high concentrations. The tumor promoter TPA, however, stimulates the proliferation and differentiation of normal unipotent (erythroid; macrophage) and bipotent (erythroid/megakaryocyte; granulocyte/macrophage) hemopoietic progenitors but has no effect on the multipotential progenitors and the undifferentiated blast cells. The modulating effect of TPA on the late progenitor cells appears to be mediated via altering the responsiveness of progenitor cells to growth regulators or by inducing accessory cells to secrete growth factors rather than acting directly on the progenitor cells.

(4) We are currently investigating the in vivo effect of DMBA and TMBA on murine hemopoietic target cells by determining the colony formation ability in cell culture of hemopoietic progenitor cells isolated from treated animals.

(5) Administration of [ring-3H]-DMBA to Long-Evans rats in single intravenous doses resulted in substantial binding of DMBA to DNA isolated from hemopoietic tissues including bone marrow and spleen. DMBA-DNA adducts were found to persist in the spleen but rapidly lost from the bone marrow. Structural confirmation of individual DMBA-DNA adducts are presently characterized.

Significance to Biomedical Research and the Program of the Institute:

This study is aimed at developing new approaches toward a better understanding of chemical carcinogenesis. The hemopoietic system offers an extremely valuable model by allowing one to study the direct effect of leukemogenic carcinogens on specific target cells.

Proposed Course:

Continue the course outlined under Objectives and Major Findings.

Publications:

Vu, V. T., Grantham, P. H., Roller, P. P., Hankins, W. D., Wirth, P. J., and Thorgeirsson, S. S.: Formation of DNA adducts from N-acetoxy-2-acetylaminofluorene and N-hydroxy-acetylaminofluorene in rat hemopoietic tissues in vivo. Cancer Res. (In Press)

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05378-02 LEC

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Hormone-Dependent Transcriptional Regulatory Elements: Structure and Function

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	Gordon L. Hager	Head, Hormone Action & Oncogenesis Section	LEC	NCI
Others:	Alexander Lichtler	Guest Researcher	LEC	NCI
	Diana S. Berard	Microbiologist	LEC	NCI
	Michael Cordingley	Visiting Fellow	LEC	NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Experimental Carcinogenesis

SECTION

Hormone Action and Oncogenesis Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.6	1.4	0.2

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The glucocorticoid hormone regulatory sequences in the mouse mammary tumor virus (MMTV) long terminal repeat (LTR) have been localized between 100 and 200 nucleotides upstream from the cap site in the LTR. Our earlier experiments (see project # Z01CP04986-08) suggested that these sequences conferred a negative effect on transcription in the absence of hormone. Other experiments (see project #Z01CP-05450-01) show that nucleosomes are phased in the vicinity of the hormone regulatory sites, indicating that the region is highly organized at the chromatin level. These results predict that deletion mutagenesis would have multiple effects, perturbing both binding sites for regulatory factors and the underlying nucleoprotein structure. We have developed a new method, "oligo-scanning mutagenesis," that permits high resolution probing of DNA-protein interactions in the region without major alteration in the overall structure. This technique permits the oligonucleotide-directed introduction of alterations in DNA sequence as large as 10 nucleotides in a one-step procedure. The method is precise (recovered mutants contain only the predicted alteration) and efficient (predicted mutants routinely represent 3-10% of recovered clones). A series of mutations induced with this technique confirm the earlier results obtained with deletion mutagenesis. That is, mutant combinations that significantly perturb the hormone response region lead to an increased constitutive level of transcription in the absence of hormone. These results indicate the hormone response is complex, involving both negative effects minus activated receptor and positive effects in the presence of hormone.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

Gordon L. Hager	Head, Hormone Action & Oncogenesis Section	LEC	NCI
Alexander Lichtler	Guest Researcher	LEC	NCI
Diana S. Berard	Microbiologist	LEC	NCI
Michael Cordingley	Visiting Fellow	LEC	NCI

Objectives:

Analysis of hormone regulated transcription of mouse mammary tumor virus (MMTV). Localization of hormone regulatory sequences involved in this regulation.

High-resolution mutagenesis of sequences involved in hormone regulation for the purpose of characterizing elements responsible for the interaction of the glucocorticoid receptor complex with the chromatin target, and to characterize the mechanism by which the rate of transcription is modulated.

Methods Employed:

Molecular chimeras between the MMTV long terminal repeat (LTR) and the v-ras gene of Harvey murine sarcoma virus (HaMuSv) will be used in a hormone-dependent transfection assay to probe the regulatory regions involved in hormone induction of MMTV expression.

This transfection assay will be adapted to the M13 single-stranded virus system to permit efficient and directed isolation of appropriate mutations.

Heteroduplex molecules will be prepared containing gaps to permit site-directed mutagenesis of regions of interest.

Mutants will be introduced into regions previously identified as necessary for the hormone response by oligo-directed mutagenesis and single-strand specific chemical mutagenesis.

Major Findings:

A new technology has been derived for the rapid isolation of extensively mismatched mutants in regions of interest. Evidence from physical characterization of nucleoprotein structure at the hormone-regulated MMTV promoter indicates that chromatin is highly organized in this region. Deletion mutagenesis thus suffers a serious weakness in that large-scale removal of sequences probably alters several parameters of structure simultaneously. Alternatively, single-base mutants usually do not impair the structure sufficiently to score a clear phenotype in the test system.

In the new methodology, called "oligo-scanning mutagenesis," the v-ras MMTV fusion system has been transferred into the M13 bacteriophage, permitting high-resolution site-directed mutagenesis in gapped molecules created by heteroduplex

formation between single-stranded molecules containing complete LTR regions and duplex molecules deleted for selected areas of the regulatory sequence. The test system, after transfer to the M13 vector, remains highly inducible to the action of glucocorticoid hormones, manifesting a 200-fold response in the number of foci recovered in the presence of hormone compared to the absence of hormone. Furthermore, S1 nuclease analysis indicates that transcription is initiated at the correct correct cap site in the test molecules and that intracellular RNA levels respond normally to hormone regulation.

The new technique permits the simultaneous change of as many as 10 base pairs without changing the relative position of non-mutated sequences with respect to each other, or with respect to other elements of the promoter.

Results obtained with a preliminary set of mutant combinations indicate: (1) that elimination of individual binding sites for the receptor complex leads to only partial impairment of the hormone response, and (2) major disruption of the hormone response region results in a large increase in the constitutive level of transcription in the absence of hormone. Therefore, the negative effect on transcription associated with the MMTV LTR by earlier deletion experiments (see project # Z01CP04986-08) has now been shown to be mediated through the same set of sequences that encode the receptor-binding sites. A long-range, high-resolution analysis of this region to characterize the mechanism of the positive and negative aspects of hormone regulation is underway.

Significance to Biomedical Research and the Program of the Institute:

Experimentation in the past five years clearly indicates that the abberant expression of genetic information, particularly elements referred to as oncogenes, is a major determinant in the development of neoplastic transformation, both in animal and in human systems. A detailed knowledge of the mechanisms involved in the control of mammalian genes is therefore paramount in the effort to understand and control cancer. The experiments conducted in this project will aid considerably in our understanding of how hormones regulate gene expression in mammalian tissues.

Proposed Course:

Oligo-scanning mutagenesis will be extended with the M13 MMTV LTR v-ras fusion system to precisely characterize sequence elements required for the hormone response. Attempts will be made to uncouple positive action of the regulatory region from the negative effect.

High-resolution mutants in the hormone response region will be transferred into amplification vectors for study in cell-free binding to the glucocorticoid receptor.

High-resolution mutants in the hormone response region will be transferred into bovine papilloma virus episomal fusions containing the MMTV LTR driving the v-ras sequence. The effect of mutants on the hormone response can therefore be tested in the normal chromatin environment, as opposed to naked DNA.

Publications:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05379-02 LEC

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT (*80 characters or less. Title must fit on one line between the borders.*)

Analysis of Polypeptide Changes During Cellular Differentiation and Transformation

PRINCIPAL INVESTIGATOR (*List other professional personnel below the Principal Investigator.*) (*Name, title, laboratory, and institute affiliation*)

PI:	Peter J. Wirth	Expert	LEC	NCI
Others:	Timothy Benjamin	Chemist	LEC	NCI
	Dolores M. Schwartz	Biologist	LEC	NCI
	Snorri S. Thorgeirsson	Chief	LEC	NCI
	Stuart H. Yuspa	Chief	LCCTP	NCI
	Henry Hennings	Senior Chemist	LCCTP	NCI
	Curtis Harris	Chief	LHC	NCI
	B. I. Gerwin	Staff Fellow	LHC	NCI

COOPERATING UNITS (*if any*)

None

LAB/BRANCH

Laboratory of Experimental Carcinogenesis

SECTION

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.3	0.6	0.7

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK (*Use standard unreduced type. Do not exceed the space provided.*)

This project was initiated to analyze, both qualitatively and quantitatively, changes in total cellular protein patterns during cellular differentiation and transformation using the technique of quantitative two-dimensional electrophoresis. Results obtained to date include: 1) Mouse epidermal cells were cultured in the presence of either low calcium (0.02-0.1 mM) (mainly basal cells) or high calcium (1.2 mM) (mainly mature keratinocytes) and then treated with 12-O-tetradecanoyl-13-acetate (TPA) for 1, 4 or 24 hours. Differentiation of epidermal basal cells were induced by increasing Ca+2 from 0.05 to 1.2 mM in the medium or by treatment with TPA. Analysis of epidermal polypeptides by 2-D electrophoresis indicated that the synthesis of eight polypeptides were increased and six decreased, and the phosphorylation of at least one polypeptide is altered similarly by Ca+2 and TPA. These common polypeptide changes, both associated with differentiation induced by Ca+2 and TPA, may involve the phorbol ester receptor, protein kinase C. 2) Analysis of polypeptide changes from normal bronchial epithelial cells (NHBE) and the Harvey v-ras transfected lines, TBE-1, TBE-1SA, and TBE-1SAT revealed the progressive appearance of four new polypeptides (pI 5.5-5.75/56-55 kDa; 4.9/55 kDa; 6.4/66 kDa; and 6.7/39 kDa) from NHBE to the most malignant TBE-1SAT line. Numerous quantitative changes were also observed, most notably, the progressive loss of expression of two polypeptides (5.6/40 kDa and 5.7/40 kDa), which were almost completely absent in TBE-1SAT cells. 3) Sodium bisulfite transformed hamster fetal cells were characterized by the shift of two polypeptides 1 and 2 (pI 5.1/52 kDa and 5.1/28 kDa), the expression of two new polypeptides 3 and 4 (6.8/44 kDa and 5.5/46 kDa) and the loss of one polypeptide 5 (6.0/55 kDa). Early polypeptide changes were analyzed in a series of 20 bisulfite transformed lines. Polypeptide changes in 1, 2, and 5 were associated with early steps in the transformation process and related to morphologic changes, whereas polypeptides 3 and 4 appear to occur later and are more closely associated with the acquisition of tumorigenicity.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

Peter J. Wirth	Expert	LEC	NCI
Timothy Benjamin	Chemist	LEC	NCI
Dolores M. Schwartz	Biologist	LEC	NCI
Snorri S. Thorgeirsson	Chief	LEC	NCI
Stuart H. Yuspa	Chief	LCCTP	NCI
Henry Hennings	Sr. Chemist	LCCTP	NCI
Curtis Harris	Chief	LHC	NCI
B. I. Gerwin	Staff Fellow	LHC	NCI
Joseph A. DiPaolo	Chief	LB	NCI
J. Doniger	Chemist	LB	NCI

Objectives:

The overall objective of this project is to employ the computer based two dimensional electrophoresis of total cellular polypeptides to analyze, both qualitatively and quantitatively, the changes in the polypeptide patterns during cellular transformation and differentiation. We plan to use this experimental technique to critically examine the hypothesis that neoplasia results from a "block" in normal cellular differentiation. The experimental systems that we are currently examining include cultured mouse epidermal cells (keratinocytes), human bronchial epithelial cells, and Syrian hamster fetal cells (HFC) in the study of blocked differentiation versus neoplastic transformation.

Methods Employed:

The principle methods employed are: 1) tissue culture techniques; 2) histochemical staining; 3) differential centrifugation; 4) autoradiography and fluorography; 5) two-dimensional electrophoresis; and 6) computer-assisted quantitation of autoradiograms and silver stained gels.

Major Findings:

1) In the cultured mouse epidermal cell system (cultured keratinocytes) Ca^{+2} is a critical regulator of growth and differentiation. In certain other cell types, a synergism exists between 12-0-tetradecanoylphorbol-13-acetate (TPA) and Ca^{+2} in the stimulation of proliferation. Ca^{+2} induced differentiation of keratinocytes is enhanced by concurrent treatment with TPA. Structural-activity studies indicate that receptor binding of phorbol esters (i.e., TPA) is required for induction of epidermal differentiation. Thus phorbol ester receptor (protein kinase C) may be a regulator of keratinocyte differentiation. If Ca^{+2} and TPA induce epidermal differentiation via a common pathway (i.e., involving protein kinase C), one would expect certain common patterns of polypeptide expression and phosphorylation following treatment with these inducers.

Mouse epidermal cells were cultured in the presence of either low (0.02-0.09 mM) or high (1.2 mM) Ca^{+2} and then treated with the TPA (0.01 and 0.1 μg/ml) for 1, 4, and 24 hours. Cells were then pulse-labeled with [14C] amino acids for 4

hours (except 1 hour TPA treated cells which were labeled for only 1 hour). Two dimensional electrophoretic analysis of total cellular polypeptides from epidermal cells grown in the presence of either low calcium (mainly basal cells) or high calcium (mainly keratinocytes) revealed only quantitative polypeptide differences. From 600-1200 polypeptides were visible on 2D gels over the pH range 4.9-7.3 and molecular weight range of 15-130 kDa. The pattern of polypeptide synthesis 1-4 hours after TPA treatment in low Ca^{+2} medium was compared to the pattern of untreated cells or those shifted to high Ca^{+2} medium. TPA treatment effected the synthesis of 122 polypeptides by two-fold or more within one hour compared to untreated low Ca^{+2} controls; 1.4 mM Ca^{+2} resulted in a two-fold or more change in 63 polypeptides compared to untreated low Ca^{+2} controls. Of the polypeptides which were modulated, the rate of synthesis of 11 were altered by both Ca^{+2} and TPA; 7 were up-regulated and 4 were down-regulated, with changes in the same direction for both differentiating agents. This result suggests that a common program of protein synthesis is induced by both Ca^{+2} and TPA, and these proteins (polypeptides) are probably related to epidermal differentiation. One polypeptide (pI 6.0/MW 80 kDa) was increased (6-8 fold) by both Ca^{+2} and TPA.

The results of 2-D gel analysis at 4 hours after TPA or Ca^{+2} were similar to those found at one hour. TPA-treated cells showed a change of three-fold or more in the synthesis of 82 polypeptides. Of these, 14 were in common with Ca^{+2}. The synthesis of 8 polypeptides increased and 6 decreased after either Ca^{+2} or TPA treatment. Again, the synthesis of one polypeptide (pI 6.0/80 kDa) was increased 7-8 fold by both Ca^{+2} and TPA. In addition to the common polypeptide changes induced by both Ca^{+2} and TPA, numerous changes specific to either Ca^{+2} or TPA treatment were noted. For example, 4 hours after TPA treatment, the synthesis of one polypeptide (pI 6.1/MW 90 kDa) is increased nearly 55-fold to become the fourth most abundant polypeptide expressed. The expression of this polypeptide is unaffected by high Ca^{+2} growth conditions.

In a preliminary phosphorylation experiment, [^{32}P]-labelled polypeptides were compared after TPA treatment (30 min) or exposure to 1.4 mM Ca^{+2}. Prior to 2-D electrophoresis, cells were fractionated into crude membrane and cytosolic preparations. No new or unique phosphorylated polypeptides were observed after either Ca^{+2} or TPA. Keratin polypeptides, present only in the membrane preparations, were heavily phosphorylated in low Ca^{+2} medium, but the level or pattern was not altered significantly by Ca^{+2} or TPA treatment. The phosphorylation of four polypeptides was increased by TPA and one of these was also increased by Ca^{+2}. The phosphorylation of two 30 kDa polypeptides with pIs of 5.8 and 6.0 (which may represent the same polypeptide with different degrees of phosphorylation) was increased 2-3 fold by TPA but only slightly by Ca^{+2}. Phosphorylation of a 42 kDa membrane polypeptide with a pI of 5.3 was increased 2-3 fold by both TPA and Ca^{+2}. The phosphorylation of a 73 kDa polypeptide was unaffected by Ca^{+2} but increased by TPA in membrane fractions. The degree of increased phosphorylation of this polypeptide is probably an underestimate, since increases in the level of phosphorylation of a group of 73 kDa polypeptides at pIs 5.75, 5.85, and 5.90 were also observed in the cytosolic fraction. This polypeptide and the two 30 kDa polypeptides were observed in both membrane and cytosolic fractions.

2) Normal human bronchial epithelial cells (NHBE) can be induced to undergo terminal squamous differentiation (including an increase in cell surface area, formation of cross-linked envelopes, and cessation of cell division) in the

presence of TPA (3 nM) or certain blood derived serum (BDS) growth factors. In contrast, however, various lung carcinoma cell lines and Harvey v-ras DNA transfected NHBE (TBE-1 cells) are resistant to induction of terminal squamous differentiation by either BDS or 100 nM TPA. These results and the work of others suggest that an aberrant control of normal differentiation is positively correlated with malignant transformation (i.e., malignant cells have a reduced capacity to respond to factors which induce terminal differentiation in normal cells).

2-D electrophoresis of [^{14}C]-labeled polypeptides from NHBE, TBE-1, and the progressively more malignant lines, TBE-1SA (TBE-1 cells selected for anchorage-independent growth), and TBE-1SAT (cells derived from TBE-1SA tumors) revealed very similar polypeptide patterns although both qualitative and quantitative polypeptide differences were observed among the various cell lines. Comparison of polypeptide patterns from normal NHBE and TBE-1 cells revealed only one qualitative difference. In TBE-1 a group of four polypeptides (probably glycosylated variants) (pI 5.5-5.7/MW 56-55 kDa) appeared. In control NHBE cells one minor polypeptide occurred at pI 5.7/55 kDa. Minor quantitative differences were also noted. No qualitative polypeptide differences were noted between TBE-1 and TBE-1SA although there were marked differences in the expression of five polypeptides: three polypeptides (pI 6.5/41 kDa; 6.6/40 kDa; and 6.7/39 kDa) were greatly increased while two polypeptides (5.6/40 kDa and 5.7/40 kDa) were decreased in TBE-1SA as compared to TBE-1 cells. Comparison of TBE-1SAT cells with TBE-1 cells showed at least three qualitative polypeptide differences and more numerous quantitative changes. New (or at least modified) polypeptides (pI 4.9/55 kDa; 6.4/66 kDa; and 6.25/58 kDa) were identified. Most prominent quantitative changes include marked increases in the expression of two polypeptides (pI 5.2/30 kDa and 6.9/26 kDa) and almost complete loss of expression of polypeptides (pI 5.6/40 kDa and 5.7/40 kDa).

NHBE, TBE-1, and A1146 (lung carcinoma cell lines) were exposed to either 10 nM TPA or TGF-B (10 ng/ml) for 24 and 72 hours in LHC-8 medium and labeled for 24 hours with [^{14}C] amino acids (normal four hour labeling period was not sufficient for effective incorporation of radioactivity; therefore, the labeling period was increased). We are currently in the process of analyzing the 2-D electrophoretograms of the polypeptides from these cell types and treatments.

3) The Syrian hamster fetal cell (HFC) offers a very attractive model for the in vitro study of transformation (neoplastic and malignant) by a wide variety of chemical and nonchemical agents. At neutral pH, sodium bisulfite ($NaHSO_3$) induces dose dependent morphological transformation of HFC in the absence of detectable DNA damage. These bisulfite transformed lines formed anchorage independent colonies in soft agar and produced progressively growing fibrosarcomas in nude mice. Two-dimensional electrophoretic analysis of [^{14}C] labeled polypeptides from $NaHSO_3$ induced transformed cells showed both qualitative and quantitative polypeptide differences. Seven neoplastic lines (A, D, E, F, G, H, I) had the same qualitative changes: polypeptides 1 and 2 (pI 5.1/52 kDa and 5.1/28 kDa) were shifted slightly to the acidic side; polypeptides 3 (6.8/44 kDa) and 4 (5.5/46 kDa) were new (except in line A); and polypeptide 5 (6.0/55 kDa) was missing. None of these changes was observed either 0 or 48 hours after $NaHSO_3$ treatment (10 μg/ml). The transformed $NaHSO_3$ lines differed quantitatively from untreated HFC in that 10-25% and 2-4% of the polypeptides exhibited differences

in expression greater than two- and four-fold, respectively. Furthermore, there were 21 specific polypeptides with coordinate quantitative changes in all transformed lines. Eleven polypeptides were down-regulated while 10 were up-regulated. These polypeptides were located throughout the gel with respect to MW and pI. However, six of these polypeptides were consistently localized in areas where the tropomyosins (pI 5.0-5.8/32-38 kDa) and actin (pI 6.1/45 kDa) are located.

Line A (20 to 25 population doublings after colony isolation) induced a tumor in only 1 or 12 nu/nu mice. The other six lines induced tumors in a minimum of 50% of the injected mice. After recloning in soft agarose, the subline A1 (MBSBA1) induced tumors in 100% of the injected mice. In lines A, A1, and TA1 (tumor derived) polypeptides 1 and 2 were shifted, polypeptide 3 was present, and polypeptide 5 was missing. Polypeptide 4 was found in lines A1 and TA1 but not in A. Therefore, the expression of polypeptide 4 appears to be associated with increased tumorgenicity.

Early polypeptide changes were determined in a series of 20 $NaHSO_3$ induced morphologic transformed colonies two days after isolation. Polypeptides 1 and 2 were always shifted and polypeptide 5 was missing. Polypeptide 3 was present in 7 of the colonies, and polypeptide 4 was present in 14 of the clones. Some clones had neither 3 nor 4, both 3 and 4, or only 3 or 4. Thus polypeptide changes in 1, 2, and 5 are associated with early steps in the transformation process and related to morpholgic changes, whereas polypeptides 3 and 4 appear to occur later and are more closely associated with acquisition of tumorigenicity.

Significance to Biomedical Research and the Program of the Institute:

The cultured mouse epidermal, human bronchial epithelial, and the Syrian hamster fetal cell systems offer very attractive models for the *in vitro* study of differentiation and transformation. It is hoped that analysis of polypeptide changes accompanying cellular transformation and normal differentiation will allow us to critically evaluate the hypothesis that neoplasia results from a "block(s)" in normal cellular differentiation.

Proposed Course:

Continue the course outlined under Objectives and Major Findings.

Publications:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05446-01 LEC

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT (*80 characters or less. Title must fit on one line between the borders.*)

Initiation and Termination of Hepatocyte Proliferation by Serum Factors

PRINCIPAL INVESTIGATOR (*List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

	Name	Title	Lab	Institute
PI:	Anthony C. Huggett	Visiting Fellow	LEC	NCI
Others:	Min-Kyung Song	Visiting Fellow	LEC	NCI
	Peter P. Roller	Chief, Biopolymer Chemistry Section	LEC	NCI
	Snorri S. Thorgeirsson	Chief	LEC	NCI

COOPERATING UNITS (*if any*)

None

LAB/BRANCH

Laboratory of Experimental Carcinogenesis

SECTION

Biopolymer Chemistry Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.6	0.6	0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK (*Use standard unreduced type. Do not exceed the space provided.*)

The purpose of this new project is to identify the serum factors responsible for the stimulation and termination of hepatocyte proliferation following partial hepatectomy of rats. At present the research is focused on the purification and biological and structural characterization of a putative polypeptide isolated from the serum of partially hepatectomized Fischer 344 male rats. A factor of molecular weight 70-120 kD which produces a dose-dependent stimulation of DNA synthesis in cultures of primary hepatocytes has been partially purified using gel filtration and heparin-affinity chromatography. The stimulatory factor is stable for over a month at -70°C. Some activity is lost by treatment with acid at pH 4 followed by dialysis (10 kD mw-cutoff) suggesting the factor may be bound to a large mw macromolecule. The partially purified serum component is also active in cultures of normal rat kidney cells, but is not active in cultures on nonmalignant liver cells isolated from a 12-day old male Fischer rat or in cultures of hepatoma cell lines. Further purification and characterization of this serum component is currently being undertaken. In addition, the investigation of serum obtained from hepatectomized rats for the presence of further stimulatory components or absence of inhibitory factors will be continued. These studies will be extended by investigating conditioned medium from hepatoma cell lines for the presence of similar growth-stimulating polypeptides in order to examine the possible involvement of the serum factors in the neoplastic process.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

Anthony C. Huggett	Visiting Fellow	LEC	NCI
Min-Kyung Song	Visiting Fellow	LEC	NCI
Peter P. Roller	Head, Biopolymer Chemistry Section	LEC	NCI
Snorri S. Thorgeirsson	Chief	LEC	NCI

Objectives:

The objective of this project is to isolate and characterize, both biologically and structurally, the serum polypeptides and proteins involved in the stimulation and termination of hepatocyte proliferation following partial hepatectomy. The aim is to investigate the role played by such factors in the neoplastic process.

Methods Employed:

The principal methods employed in these studies include: (1) use of affinity chromatography, ultrafiltration, fast protein liquid chromatography (FPLC), high performance liquid chromatography and two-dimensional polyacrylamide gel electrophoresis to purify polypeptides and proteins from serum obtained from partially hepatectomized Fischer rats; (2) maintenance of primary hepatocyte cultures in defined serum-free medium; (3) fluorimetric assay of cellular DNA and assay of the incorporation of tritiated thymidine into DNA of cells maintained in 96-well microtiter plates; (4) study of the action of these factors on various cell types including normal hepatocytes, preneoplastic and neoplastic hepatocytes and cells from other tissues.

Major Findings:

Previous studies in other laboratories have shown that serum obtained from partially hepatectomized rats is more active in stimulating DNA synthesis in primary hepatocyte cultures than serum obtained from control rats. We are investigating serum from hepatectomized rats in order to determine the components responsible for this difference. We have achieved a partial purification of a factor which stimulates DNA synthesis in primary cultures of rat hepatocytes using heparin-affinity chromatography and gel filtration of serum from Fischer rats 24 hours after two-thirds partial hepatectomy. A 6 to 7-fold increase in DNA synthesis compared to control was observed on addition of the partially purified serum factor (10 μl) to the primary hepatocyte cultures (100 μl). The stimulation was dose-dependent. A similar increase in DNA synthesis (6 to 7-fold) was observed on addition of EGF (10 ng/ml) plus insulin (0.1 μM) to the hepatocyte cultures. In contrast the growth factors PDGF and EGF produced no stimulation of DNA synthesis in these cell cultures. The partially purified serum factor has an apparent molecular weight of 70,000-120,000 daltons and is stable for at least two months at -70°C. Some activity is lost by treatment with acid at pH 4 followed by dialysis (10,000 mw-cutoff). This stimulatory factor is also active in cultures of normal rat kidney cells, but is not active in cultures of

cultures of hepatoma cell lines. Similar results were obtained when EGF was tested in these cell cultures.

Significance to Biomedical Research and the Program of the Institute:

Our studies are aimed at identifying regulatory protein factors involved in hepatocyte proliferation. The results of these studies could provide an insight into differences in the growth control mechanisms of normal cells compared with neoplastic cells.

Proposed Course:

Complete purification and characterization of the stimulatory serum factor (mw 70-120 K daltons) will be undertaken. In addition, the investigation of serum obtained from hepatectomized rats for the presence of further stimulatory components or the absence of inhibitory factors will be continued. Previous studies in this laboratory have shown that following a partial hepatectomy, a regenerative stimulatory activity is produced which is transmitted through the circulation and stimulates the proliferation of hepatocytes transplanted to the anterior chamber of the eye. Eye fluid obtained from partially hepatectomized rats will be examined in order to elucidate the nature of this regenerative signal. The role played by known polypeptide modulators of hepatocyte growth (e.g., EGF, insulin, IGF-I, IGF-II) in the regeneration of liver following partial hepatectomy will also be investigated. These studies will be extended by investigating conditioned medium from hepatoma cell lines for the presence of similar growth stimulating polypeptides in order to examine the possible involvement of the serum factors in the neoplastic process.

Publications:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01CP05447-01 LEC

PERIOD COVERED
October 1, 1984 to September 30, 1985

TITLE OF PROJECT (*80 characters or less. Title must fit on one line between the borders.*)
Isolation and Characterization of Proteins from Two-Dimensional Polyacrylamide Gels

PRINCIPAL INVESTIGATOR (*List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Anthony C. Huggett	Visiting Fellow	LEC	NCI
Others:	Timothy Benjamin	Chemist	LEC	NCI
	James L. Cone	Chemist	LEC	NCI
	Preston H. Grantham	Chemist	LEC	NCI
	Henry C. Krutzsch	Expert	LEC	NCI
	Peter J. Wirth	Expert	LEC	NCI
	Peter P. Roller	Head, Biopolymer Chemistry Section	LEC	NCI
	Snorri S. Thorgeirsson	Chief	LEC	NCI

COOPERATING UNITS (*if any*)

None

LAB/BRANCH
Laboratory of Experimental Carcinogenesis

SECTION
Biopolymer Chemistry Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.8	0.5	0.3

CHECK APPROPRIATE BOX(ES)
☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (*Use standard unreduced type. Do not exceed the space provided.*)

The purpose of this project is to develop the novel analytical technology required for the elution and microsequencing of proteins from two-dimensional polyacrylamide gels. The availability of such a technique will allow the construction of DNA probes in order that both gene cloning and studies on gene expression can be achieved. This will enable studies of the regulation of gene expression at the transcription, translation and post-translation levels to be undertaken. Protein sequence information will permit the chemical synthesis of small peptides which will be used for the production of antibodies. These will greatly facilitate the large scale purification of the protein and also aid in their identification and quantitation. Initial investigations have been focused on the development of microscale procedures for sample handling, enzymic digestion and peptide purification by reverse-phase HPLC. Electroelution of proteins from polyacrylamide gels was studied using I-125 labeled proteins. As little as 15 pmoles of protein could be recovered in good yield (> 60%) from coomassie blue stained gels. In contrast the yield of protein from silver stained gels was poor. The feasibility of protein extraction by transblotting onto nitrocellulose followed by solubilization of the nitrocellulose is currently being evaluated. Methodology for the microscale trypsin digestion of proteins, followed by HPLC of the peptide fragments, is also being developed. Preliminary studies using carbonic anhydrase as a model peptide have been successful and amino acid analysis of the tryptic peptides was performed. Work is presently underway to sequence microquantities of these peptides using gas chromatography combined with electron impact mass spectrometry.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

Anthony C. Huggett	Visiting Fellow	LEC	NCI
Timothy Benjamin	Chemist	LEC	NCI
James L. Cone	Chemist	LEC	NCI
Preston H. Grantham	Chemist	LEC	NCI
Henry C. Krutzsch	Expert	LEC	NCI
Peter J. Wirth	Expert	LEC	NCI
Peter P. Roller	Head, Biopolymer Chemistry Section	LEC	NCI
Snorri S. Thorgeirsson	Chief	LEC	NCI

Objectives:

The major objective of this project is to isolate and characterize proteins from polyacrylamide gels following two-dimensional polyacrylamide gel electrophoresis of relatively crude tissue preparations. The availability of partial protein sequence information will allow the construction of DNA probes which will facilitate both gene cloning and studies on gene expression. Techniques for the microsequencing of the trace quantities of protein obtainable from a two-dimensional gel protein spot are unavailable at present so the initial objective of this project is to develop the micro-analytical technology required.

Methods Employed:

The principal methods employed are: (1) two-dimensional polyacrylamide gel electrophoresis; (2) transblotting of proteins to nitrocellulose; (3) electro-elution of proteins from two-dimensional gels; (4) high performance liquid chromatography; (5) chemical and enzymic digestion of proteins; (6) amino acid analysis of proteins; and (7) microsequencing of proteins using gas chromatography with electron impact mass spectrometry.

Major Findings:

The computer-assisted two-dimensional gel electrophoresis methodology developed within this laboratory has provided a highly sensitive procedure capable of resolving large numbers of proteins within a sample. This technique has been successfully applied to the investigation of differences in gene expression between normal, preneoplastic and neoplastic tissues. These studies have demonstrated qualitative as well as quantitative alterations in protein profiles. Of particular interest are those proteins which are produced in preneoplastic and neoplastic tissue but not in normal tissue. We have focused our studies on the differences in protein patterns obtained from 2D-PAGE analysis of Solt-Farber induced neoplastic hepatic nodules with the patterns obtained from analysis of normal hepatic tissue. At least four protein spots have been detected in neoplastic tissue which were not found in normal tissue (mol. wt., pI, location): (A) 57 kD, 6.8, cytosol; (B) 41 kD, 6.25, membrane; (C) 26 kD, 6.75, membrane; (D) 24 kD, 6.05, membrane.

The isolation and characterization of these proteins requires the development of a battery of microanalytical techniques due to the small quantity of protein present in a two-dimensional gel spot. Initial investigations have been focused on the development of microscale procedures for sample handling, enzymic digestion and peptide purification. In addition, classical procedures for peptide and protein sequencing involving Edman degradation cannot be applied to proteins and peptides whose amino-terminus is blocked and are also not applicable for the analysis of trace amounts of sample. Therefore novel microsequencing techniques are being investigated.

Initial studies concerning the electroelution of I-125 labeled proteins from polyacrylamide gels have demonstrated a poor recovery of protein from silver stained gels. In contrast, protein elution following visualization with coomassie blue dye was successful in that between 65% and 95% of the protein could be recovered depending on the protein used (albumin, carbonic anhydrase or trypsin inhibitor) and the amount applied to the gel (15-1000 pmoles). Amino acid analysis of the resulting protein solution has thus far been unsuccessful due to contamination of the sample with amino acids and other components also eluted from the gel.

Methodology for the trypsin digestion of pmole amounts of proteins is currently being developed. Preliminary studies using carbonic anhydrase as a model protein have been successful and amino acid analysis of the tryptic peptides separated by reverse-phase HPLC was performed. Work is presently underway to modify the dipeptidase method of peptide sequencing using electron impact mass spectrometry devised by Krutzsch (1983) to allow sequencing of these trypic peptides.

Significance to Biomedical Research and the Program of the Institute:

Knowledge of the biochemical changes associated with the neoplastic process is a prerequisite for the understanding of the mechanisms responsible for the onset and progression of the malignant phenotype. Our studies are aimed at identifying changes in gene expression occurring as a result of chemical transformation and to develop methodology in order to characterize, both structurally and biochemically, the proteins that are highly associated with tumorigenesis. The methodology will have universal applicability to the isolation, sequencing and identification of sub-nanomole amounts of proteins from virtually any source.

Proposed Course:

The elution of sub-nanomole amounts of proteins from polyacrylamide gels will be continued as outlined under Major Findings. Methodology for the purification of the eluted protein prior to amino acid analysis will be developed. The feasibility of protein extraction from polyacrylamide gels by transblotting onto nitrocellulose followed by solubilization of the nitrocellulose will be examined. The development of microsequencing techniques for the analysis of N-terminal blocked peptides will be continued.

The successful elution of proteins from two-dimensional gels and sequencing of peptide fragments will allow the production of specific DNA probes. These will be used to facilitate cloning and sequencing of the genes. In addition, the probes will permit the isolation of mRNAs coding for the proteins such that

studies of their regulation at the transcription, translation and post-translation levels can be undertaken.

A knowledge of partial sequence information of the proteins will greatly simplify the purification of larger amounts by more conventional procedures. Small peptides (10-20 residues), corresponding to the sequences of the tryptic peptides, will be chemically synthesized and used for the production of monoclonal antibodies. These antibodies will be immobilized onto chromatography columns and used for protein purification. In addition, the development of antibodies will greatly aid in the identification and quantitation of the proteins under investigation.

Publications:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05448-01 LEC

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT (*80 characters or less. Title must fit on one line between the borders.*)

Guanosine Triphosphate Binding Site of Ras Protein by NMR and CD Spectroscopy

PRINCIPAL INVESTIGATOR (*List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Chien-Hua Niu	Expert	LEC	NCI
Others:	Kyou-Hoon Han	Visiting Fellow	LEC	NCI
	Peter P. Roller	Head, Biopolymer Chemistry Section	LEC	NCI

COOPERATING UNITS (*if any*)

None

LAB/BRANCH

Laboratory of Experimental Carcinogenesis

SECTION

Biopolymer Chemistry Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.1	1.1	0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK (*Use standard unreduced type. Do not exceed the space provided.*)

The objective of this project is to study the chemical interactions of the ras proteins (p21) and guanosine nucleotides. The guanosine triphosphate (GTP) and guanosine diphosphate (GDP) binding site of these proteins are to be precisely determined by nuclear magnetic resonance (NMR), circular dichroism (CD), equilibrium dialysis, synthesis of peptides by the solid-phase method, and calculations of binding constants and thermodynamic parameters of GTP-peptide complexes. At present, the research is focused on the following areas: (1) The solid-phase synthesis of the 34 amino acid residue N-terminal sequence of both the proto-oncogene and the oncogene of ras proteins (p21). (2) Purification of peptides by column chromatography and high performance liquid chromatography (HPLC). (3) Studies on the conformational difference between the glycine-containing (Gly-peptide) and aline-containing (Val-peptide) 34 amino acid residue-peptide by CD spectroscopy (the glycine or valine is at position 11). (4) Studies on the conformational changes of both Gly-peptide and Val-peptide upon addition of either GTP or GDP using both NMR and CD spectroscopy. (5) Studies on the interaction between peptides and GTP by NMR spectroscopy. Results obtained so far are as follows: (1) The information obtained from CD studies shows that both Gly-peptide and Val-peptide adopted predominately beta-sheet conformation (55-60%) in a non-ionic detergent solution. (2) Upon addition of GTP to the peptide solution, both Gly-peptide and Val-peptide conformation remained in the beta-sheet conformation. However, the random coil conformation gradually increases at the expense of beta-sheet conformation. (3) When the Gly-peptide was added to the GTP solution containing sodium dodecyl sulfate, the line widths of P-31 signals of both beta and gamma resonances of GTP were broadened significantly (about 10 Hz), implying that binding probably occurs between GTP and the Gly-peptide.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

Chien-Hua Niu	Expert	LEC	NCI
Kyou-Hoon Han	Visiting Fellow	LEC	NCI
Peter P. Roller	Head, Biopolymer Chemistry Section	LEC	NCI

Objectives:

The main objectives of the project are (1) to identify the guanosine triphosphate (GTP) and guanosine diphosphate (GDP) binding site in p21 proteins, (2) to study the conformational difference between proto-oncogene and oncogene product (p21), and (3) to study the conformational changes in p21 proteins upon GTP and GDP binding by spectroscopic methods. These detailed studies on p21 binding may provide important information explaining why GTP-p21 protein binding may initiate the cascade of events that ultimately results in tumorigenic transformation.

Methods Employed:

The principal methods employed are (1) peptide synthesis, (2) nuclear magnetic resonance (NMR), (3) circular dichroism (CD), (4) equilibrium dialysis, and (5) calculations of binding constants and thermodynamic parameters.

Major Findings:

1. Using hydroxybenzotriazole esters of t-butoxycarbonyl-amino acids in the solid phase synthesis provides a more economical and efficient way for synthesizing peptides than the symmetric anhydride method which has been known to prevent racemization during the coupling reaction.

2. The data obtained from CD studies of both the glycine-containing (Gly-peptide) and the valine-containing (Val-peptide) 34 amino acid residue N-terminal sequence of p21 proteins indicates that they both adopt predominately the beta-sheet conformation (60-55%) in non-ionic detergent solution. The result suggests that the secondary structure of the N-terminal sequence of p21 proteins is most likely to adopt a beta-alpha-beta conformation predicted by Hol, but unlike Feldmann's model which predicts only an alpha-helix structure.

3. Upon addition of GTP to the peptide solution, both Gly- and Val-peptide conformations remained in the beta-sheet conformation. However, the random coil conformation gradually increased at the expense of beta-sheet conformation.

4. When the Gly-peptide was added to GTP solution containing sodium dodecyl sulfate, the line widths of the P-31 signals of both beta and gamma resonance of GTP were broadened significantly (about 10 Hz), implying that the binding probably occurs between GTP and the Gly-peptide.

Significance to Biomedical Research and the Program of the Institute:

This research project is aimed at understanding the difference between proto-oncogene and oncogene protein (p21) at the molecular level. The knowledge thus obtained should provide insight into the oncogenic effects of p21.

Proposed Course:

1. Continue as outlined under Objectives and Major Findings.

2. The studies will be expanded to examine the whole p21 protein by modern spectroscopic methods.

Publications:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05449-01 LEC

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Conformational Studies on Epidermal Growth Factor and Transforming Growth Factor

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	Chien-Hua Niu	Expert	LEC	NCI
Others:	Kyou-Hoon Han	Visiting Fellow	LEC	NCI
	Peter P. Roller	Head, Biopolymer Chemistry Section	LEC	NCI
	Snorri S. Thorgeirsson	Chief	LEC	NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Experimental Carcinogenesis

SECTION

Biopolymer Chemistry Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.2	1.2	0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The purpose of this new project is to study the three dimensional structure of both beta-epidermal growth factor (beta-EGF) and transforming growth factor-type 1 (alpha-TGF) and their synthetic analogs to elucidate their biological action. The methods used include synthesis of peptides by the solid phase method, binding assays, nuclear magnetic resonance (NMR) spectroscopy, computer graphic techniques, and x-ray diffraction. The research focuses on the following areas: (1) the solid phase synthesis of the binding region of both beta-EGF and alpha-TGF; (2) cyclization of synthetic peptides to form disulfide linkages between cystein residues by a high dilution method; (3) binding assays to test their biological activities; (4) conformation studies of both synthetic beta-EGF and alpha-TGF by modern two-dimensional NMR techniques; (5) using computer graphic techniques to design possible antagonists of alpha-TGF, and synthesizing peptides for biological testing; (6) using NMR spectroscopy to study the conformation of both beta-EGF and alpha-TGF in order to determine whether the synthetic peptides resemble the natural protein conformations; and (7) x-ray studies of both beta-EGF and alpha-TGF for comparison of their crystal and solution structures. The binding regions of both beta-EGF and alpha-TGF have already been successfully synthesized. Cyclization between two cysteins will be carried out soon.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

Chien-Hua Niu	Expert	LEC	NCI
Kyou-Hoon Han	Visiting Fellow	LEC	NCI
Peter P. Roller	Head, Biopolymer Chemistry Section	LEC	NCI
Snorri S. Thorgeirsson	Chief	LEC	NCI

Objectives:

Epidermal growth factor (beta-EGF) is a single-chain polypeptide of 53 amino acid residues with three disulfide bonds. Transforming growth factor (alpha-TGF) is structurally and functionally related to beta-EGF. Both exhibit similar activities in competition for binding to the EGF receptor, stimulation of DNA synthesis, and cell growth. Available structural data on both beta-EGF and alpha-TGF should allow us to study their mitogenic activities and, from the viewpoint of conformational studies, may permit us to design antagonists against alpha-TGF for therapeutic use.

Methods Employed:

The principal methods employed are (1) peptide synthesis, (2) binding assays, (3) nuclear magnetic resonance (NMR) spectroscopy, (4) computer graphic techniques, (5) crystallization of peptides, and (6) x-ray diffraction.

Major Findings:

The syntheses of the binding regions of both beta-EGF and alpha-TGF by the solid phase method are underway.

Significance to Biomedical Research and the Program of the Institute:

This new research project is aimed at understanding the mitogenic action of both beta-EGF and alpha-TGF. The knowledge thus obtained may allow us to develop antagonists of alpha-TGF for therapeutic use.

Proposed Course:

1. To understand the biological action and locate the binding region of human beta-EGF and rat alpha-TGF. The peptide segments at binding regions will be synthesized.

2. Biological activities of synthetic peptides will be tested.

3. Conformational analysis of these synthetic peptides will be studied by NMR spectroscopy. Knowledge obtained from these studies will not only provide information on the three dimensional structures of these peptides, but should also help us to study the whole beta-EGF and alpha-TGF molecules in terms of chemical shifts of individual amino acid residues.

4. Conformational analysis of both beta-EGF and alpha-TGF proteins by either two-dimensional NMR techniques or x-ray diffraction will provide detailed information on the three dimensional structures which might allow us to develop antagonists of alpha-TGF for therapeutic use.

Publications:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05450-01 LEC

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Chromatin Structure and Steroid Hormone Action

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	Gordon L. Hager	Head, Hormone Action & Oncogenesis Section	LEC	NCI
Others:	Helene Richard-Foy	Guest Researcher	LEC	NCI
	Ronald G. Wolford	Microbiologist	LEC	NCI
	Diana S. Berard	Microbiologist	LEC	NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Experimental Carcinogenesis

SECTION

Hormone Action and Oncogenesis Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.8	1.1	0.7

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The role of chromatin organization in hormone action has been addressed utilizing chimeras between the MMTV-LTR and the bovine papilloma virus (BPV) 69% transforming fragment. These chimeras replicate uniquely as episomal elements in murine fibroblasts and maintain stable high-copy extrachromosomal copy numbers. It was found that nucleosomes are non-randomly organized on the sequences immediately upstream from the MMTV cap site and that this phased structure is independent of hormone induction. A DNase I hypersensitive site is introduced into the chromatin structure upon hormone induction; the location of this site correlates precisely with the sequences required for transfer of hormone regulation in a biological assay. Thus a highly organized nucleoprotein structure serves as a template for hormone regulation in vivo. The episomal elements can be extremely purified from cellular chromatin by selective extraction from the nuclear fraction, sedimentation through sucrose gradients, and concentration. The purified minichromosomes retain their association with 2 to 6 moles of glucocorticoid receptor subunit; this association is specific in that no bound receptor is found with control episomes not containing the MMTV LTR sequences. The fraction is highly enriched, with greater than 90% of the DNA in the fraction of episomal origin. These minichromosomes should serve as useful templates for in vitro transcription studies.

PHS 6040 (Rev. 1/84)

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

Gordon L. Hager	Head, Hormone Action & Oncogenesis Section	LEC	NCI
Helene Richard-Foy	Guest Researcher	LEC	NCI
Ronald G. Wolford	Microbiologist	LEC	NCI
Diana S. Berard	Microbiologist	LEC	NCI

Objectives:

Chimeras between bovine papilloma virus and the mouse mammary tumor virus LTR has been constructed. Cell lines are to be characterized that carry uniquely episomal chimeric DNA, and in which hormone regulation at the episomal promoter can be demonstrated.

Episomal chimeric DNA will be isolated from these cells as intact nucleoprotein particles. These particles will be used in nuclease digestion studies to characterize chromatin organization in the vicinity of the hormone regulated promoter and to identify changes in structure associated with hormone action. Binding localizations of regulatory proteins are to be determined by footprint analysis.

Mutants in the hormone response developed and characterized by oligo-scanning mutagenesis (see project # Z01CP05378-03) will be transferred to the episomal vectors and the mutational effects on chromatin structure characterized.

Methods Employed:

Minichromosomes to be used for nuclease digestion and in vitro transcription templates will be purified by low salt procedures that are unlikely to damage chromatin structure. These procedures will depend on the small size of the episomes, but they must also be able to dissociate the higher orders of structure present in the nuclear matrix including so-called nuclear scaffolding. Chelaters of specific ions may be useful in this process.

Minichromosomes used for run-off transcription are prepared from nuclei of cells containing LTR episomes by ammonium sulfate extraction. This procedure relies on the small size of these episomes as compared to chromosomal DNA. These particles are then incubated with radioactive ribonucleotides. The RNA produced is analyzed by hybridization to single-strand probes representing the sequences present in the chimeric BPV LTR episomes.

Isolation of steady-state levels of RNA and S1 mapping of this RNA using end-labeled probes are accomplished using published procedures.

Major Findings:

Nucleosome positioning on the LTR of mouse mammary tumor virus, which encodes a glucocorticoid dependent promoter, has been found to be sequence specific. An array of phased nucleosomes exists upstream of the initiation site for MMTV

transcription, beginning at position -70, and extending through to the left end of the LTR. The first nucleosome in this phased array includes sequences which are necessary for transfer of biological regulation in gene transfer experiments and which contain two binding sites for glucocorticoid receptor in vitro. The sequences which interact with the hormone regulatory molecule are therefore displayed on the surface of a phased nucleosome. This same region becomes hypersensitive to DNase I upon activation of the promoter with hormone, indicating a major alteration in chromatin structure in this regulatory region.

Significance to Biomedical Research and the Program of the Institute:

It is now widely recognized that growth factors and hormones are frequently involved in the control of both normal cell growth and abnormal neoplastic transformation. The processes by which these agents affect cell proliferation must be understood ultimately in the mechanisms by which they alter gene expression. An understanding, in turn, of the extent to which chromatin structure plays a role in hormone regulation of gene expression will have broad significance for our basic knowledge of the neoplastic process and our ability to block or reverse oncongenesis.

Proposed Course:

Two models are possible to explain the findings of this project. The alterations in chromatin structure that accompany hormone induction are a result either of the displacement of the regulatory region associated nucleosome by activated hormone receptor complex, or of the modulation of this nucleosome structure such that hypersensitivity is created. Experiments will be pursued to precisely define the complex interaction between hormone receptor and the nucleoprotein template and the alterations that occur upon hormone stimulation.

Publications:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05451-01 LEC

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Hepatocellular Carcinoma: Expression of Retroviral Associated Oncogenes

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	Brian Huber	Staff Fellow (PRAT)	LEC	NCI
Others:	Snorri S. Thorgeirsson	Chief	LEC	NCI
	Carole A. Heilman	Sr. Staff Fellow	LEC	NCI
	Irene B. Glowinski	Staff Fellow	LEC	NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Experimental Carcinogenesis

SECTION

Chemical Carcinogenesis Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.2	1.2	0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The objective of this project is to characterize the role of retroviral associated oncogenes in both the formation and/or maintenance of hepatocellular carcinoma. The experimental systems presently under study are (A) a human hepatoma cell line, HEP G2 and (B) an in vivo rat model for chemically induced hepatocellular carcinoma. The results obtained with the HEP G2 study include the tumorigenic characterization of this cell line which may be associated with elevated levels of c-myc transcripts coupled to the expression of an "activated" N-ras gene. Gene expression in the progressive development of chemically induced hepatocellular carcinoma in the rat was also examined. Early preneoplastic changes were examined by isolating preneoplastic foci hepatocytes (which lack the cell-surface asialoglycoprotein receptor) in addition to later stage preneoplastic nodules and hepatocellular carcinomas. In this system, four genes are being analyzed in detail: ornithine decarboxylase (associated with proliferation); alpha-fetoprotein (associated with a less differentiated state); p53 (associated with the transformed phenotype); and myc (associated with certain human malignancies). Changes in expression of these four genes were characteristic of late stage changes rather than early premalignant changes. Finally, the modulation of the asialoglycoprotein receptor in the carcinogenic process was shown to be a post-transcriptional event in vivo.

PHS 6040 (Rev. 1/84)

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

Brian Huber	Staff Fellow (PRAT)	LEC	NCI
Snorri S. Thorgeirsson	Chief	LEC	NCI
Carole A. Heilman	Sr. Staff Fellow	LEC	NCI
Irene B. Glowinski	Staff Fellow	LEC	NCI

Objectives:

The objective of this project is to characterize the role of retroviral associated oncogenes in both the formation and/or maintenance of hepatocellular carcinoma. To realize these objectives, two experimental systems are being utilized. The first system employs a human hepatoma cell line, HEP G2, which provides a model where gene expression can be examined in two distinct, but well-defined environments, i.e., in tissue culture and in the nude mouse. The second system under study is an *in vivo* rat model for chemically induced hepatocellular carcinoma where gene expression can be investigated during the transition from normal hepatocytes to preneoplastic foci, preneoplastic nodules and neoplastic tumors. The main focus of the current research is on defining the role of c-myc and ras genes in hepatocellular carcinoma.

Methods Employed:

Methods used in these studies include: tissue culture techniques; liver perfusion; radioimmunoassay; differential centrifugation and chromatographic techniques; radioisotope measurements using tritium, carbon-14, phosphorus-32 and sulfur-35; enzyme assays involving radiometric and spectrophotometric determinations; computer-assisted two-dimensional gel electrophoresis; molecular biological techniques including Northern, Southern and Western blotting.

Major Findings:

A. The Tumorigenic Characterization and Oncogene Expression in a Human Hepatoma Cell Line, HEP G2. HEP G2 is a human cell line derived from a primary hepatocellular carcinoma. This cell line retains some biosynthetic capabilities of normal liver parenchymal cells as well as the capacity to metabolically activate a variety of chemicals, such as cyclophosphamide. We have demonstrated that this cell line is tumorigenic when injected subcutaneously into athymic nude mice, with the tumorigenicity being dependent on the number of injected cells. Tumors were nonencapsulated, highly invasive adenocarcinomas and were positive for gamma-glutamyl transpeptidase activity and bile production. Plasma from tumor bearing mice were positive for human alpha-fetoprotein indicating the human origin of the tumors, but serum was negative for hepatitis B virus surface antigen as measured by radioimmunoassay. Cyclophosphamide (CY) pretreatment of HEP G2 cells (500 μg CY/ml/two cell cycles) significantly elevated the number of sister chromatid exchanges indicating significant DNA damage, but had no effect on tumor incidence or latency when these CY pretreated cells were injected

into nude mice. Two cell lines were reestablished into tissue culture from HEP G2 derived tumors and were shown to have similar cellular morphology and unaltered cell cycle times when compared to the parent HEP G2 cell line.

This HEP G2 system, using normal human liver as a control, provides a model for identifying the genes and gene products that are associated with the tumorigenic phenotype. To demonstrate that these cells still maintain an overall genetic similarity to normal human liver, poly(A)RNA isolated from HEP G2 derived cells and tumors were in vitro translated using a cell-free rabbit reticulocyte lysate system. HEP G2 translation products, when analyzed by two-dimensional polyacrylamide gel electrophoresis, were extremely similar to the translation products from poly(A)RNA isolated from a normal human liver sample, except for one 53,000 molecular weight polypeptide with an apparent charge shift.

As a first step in identifying the genes which bestow the transformed phenotype to the HEP G2 cells, expression of proto-oncogenes (cellular oncogenes) implicated in other human malignancies was examined. C-myc specific transcripts, when compared to a normal human liver sample, were increased in all HEP G2 cell lines and tumors derived from HEP G2 cells. Contrary to other systems, the elevation in HEP G2 c-myc transcripts could not be superinduced by cycloheximide treatment. Southern blot analysis indicated that the increase in c-myc specific transcripts could not be explained by myc gene amplification or hepatitis B virus integration into the HEP G2 genome. These data suggest that transcription of the human c-myc gene is elevated in both cultured cells and transplantable tumors of HEP G2 origin. However, other possibilities such as specific stabilization of c-myc mRNA cannot be excluded.

Since DNA transfection experiments in rat embryo fibroblasts suggest that two genes from two distinct complementation groups of oncogenes or viral elements are required for cell transformation, we examined HEP G2 derived cells and tumors for the expression of an oncogene family, ras, which is complementary to the myc gene for cell transformation. C-Ha-ras expression could not be detected in any HEP G2 derived tumor or cell lines. Equal levels of 5.5 kb and 2.5 kb N-ras specific transcripts were detected in all HEP G2 derived cell lines and normal liver samples. However, RNA isolated directly from the tumors produced by HEP G2 cells had approximately a five-fold increase in the 5.5 kb N-ras specific transcript. This selective increase in the 5.5 kb N-ras transcript was reproducibly elevated in three tumors that were tested. Since this increased 5.5 kb transcript did not remain elevated if the tumors were reestablished into tissue culture, it suggested some interaction with the host animal, such as with a serum growth factor or hormone.

It is important to note, however, that an increase in N-ras specific transcripts may not be critical to complement the increased c-myc expression in tumor formation if the ras gene has been activated by a point mutation. Recent experiments have shown that the N-ras gene of HEP G2 cells is capable of transforming NIH/3T3 cells in a DNA transfection assay (Notario et al., Cancer Cells, p. 425. Cold Spring Harbor Laboratory, 1984). This indicates that the gene is activated, since unmutated N-ras genes derived from normal human cells are not capable of transforming NIH/3T3 cells in this system. Hence, normal transcriptional levels of the activated N-ras gene may complement the increased c-myc transcripts in the

tumorigenic properties of HEP G2 cells. This hypothesis is based on the assumption that the HEP G2 N-ras gene is translationally active and produces a gene product, P21, which has different properties from an unmutated N-ras gene product (i.e., GTPase activity). Immunoprecipitation of S-35 labeled HEP G2 cell lysates with anti P21 antibodies demonstrated a specific immunoprecipitable P21 polypeptide.

B. Gene Expression in the Progression of Chemically Induced Hepatocellular Carcinoma in the Rat. An important feature in the development of many if not all human and experimental tumors, including hepatocellular carcinoma, is the appearance of preneoplastic lesions which may undergo additional alterations during the progression to cancer. For this reason we have utilized an *in vivo* rat model to study gene expression sequentially throughout chemically induced liver carcinogenesis. The rat liver has been used extensively as an experimental model in carcinogenesis studies and allows identification of preneoplastic cell populations very early in the neoplastic process. To induce preneoplastic and neoplastic lesions the method of Solt-Farber was used, which includes i.p. administration of diethylnitrosamine (200 mg/kg body weight) for initiation and partial hepatectomy combined with low dose feeding of acetylaminofluorene (0.02%). An early preneoplastic alteration in hepatocytes is the appearance of focal cell populations with a greatly reduced concentration of the asialoglycoprotein receptor, a hepatic cell surface receptor which binds and internalizes desialylated serum glycoproteins, such as asialofetuin. Preneoplastic focal hepatocyte populations were isolated from collagenase perfused liver cell suspensions by their inabilty to bind to tissue culture plates coated with asialofetuin.

Gene expression was compared and contrasted in normal rat liver, preneoplastic foci, preneoplastic nodules, and primary hepatocellular carcinomas by Northern blot analysis. Initially, the expression of four genes was examined in detail: ornithine decarboxylase (associated with proliferation); alpha-fetoprotein (associated with a less differentiated state); P-53 (associated with the transformed phenotype); and myc (an oncogene associated with certain human maligancies including hepatocellular carcinoma [see above]). Compared to normal rat liver, no change in specific transcripts of any of the four genes was found in the preneoplastic foci. Preneoplastic nodules had slightly elevated ODC transcripts but all other genes remained unchanged. Neoplastic tumors had greatly elevated ODC transcripts (to the levels observed 24 hour post 70% hepatectomy), elevated P53 transcripts, just detectable AFP transcripts, and detectable transcripts which hybridize to a v-myc probe only under moderately stringent conditions. These tumors were characterized by a great deal of heterogeneity with regard to expression of the four genes analyzed.

C. Transcriptional and Post-Transcriptional Regulation of the Asialoglycoprotein Receptor (ASGPR). The ASGPR is a hepatocyte cell surface receptor which binds and internalizes serum glycoproteins with galactose-terminal carbohydrate chains. Recent experiments have demonstrated that cell-surface binding activity and cell-surface receptor proteins are decreased or absent in fetal, regenerating, phenobarbital treated and neoplastic liver, while binding activity is increased in livers of pregnant dams. We have capitalized on this phenomena to isolate preneoplastic foci in carcinogen treated rat livers (see B above). To understand more about this phenomena, we investigated the genetic regulation of the ASGPR

by isolating poly(A)RNA from fetal, neonatal, regenerating, phenobarbital treated and chemically induced neoplastic Fischer rat livers. When compared to adult liver, the amount of ASGPR specific transcripts showed no change: at 1, 3, 12, 24 and 64 hour post 70% hepatectomy; in fetal, neonatal and livers of pregnant dams; in livers of phenobarbital treated rats; and in chemically induced (Solt-Farber) preneoplastic and neoplastic liver samples. These data suggest that, *in vivo*, the ASGPR is regulated post-transcriptionally, possibly at the cell membrane. Interestingly, no transcripts could be detected in a Morris hepatoma cell line, 7777, despite being able to detect the ASGPR gene by Southern blot analysis. This indicates that in tissue culture, the ASGPR gene could be regulated at the transcriptional level. In addition, preliminary data indicates that freshly isolated hepatocytes, when placed into tissue culture, have significantly lower ASGPR specific transcripts within approximately 2 hours.

Significance to Biomedical Research and the Program of the Institute:

This project has contributed to the overall understanding of the role of retroviral associated oncogenes in neoplastic process.

Proposed Course:

Continue along lines outlined under Objectives and Major Findings.

Publications:

Huber, B. E., Dearfield, K. L., Williams, J. R., Heilman, C. A. and Thorgeirsson, S. S.: The tumorigenicity and transcriptional modulation of c-myc and N-ras oncogenes in a human hepatoma cell line. *Cancer Res.* (In Press)

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05452-01 LEC

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Gene Expression and Development in Transgenic Mice

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	Su-yun Chung	Senior Staff Fellow	LEC	NCI
Others:	Snorri S. Thorgeirsson	Chief	LEC	NCI
	Miriam Falzon	Visiting Fellow	LEC	NCI
	Nancy Sanderson	Chemist	LEC	NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Experimental Carcinogenesis

SECTION

Cell Biology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.9	1.5	0.5

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The overall objective of this project is to exploit the transgenic mouse system by introducing natural or manipulated gene sequences into the germline of an animal and to alter its phenotype and genetic background. This system provides a new way of investigating tissue specific and developmental stage specific regulation of gene expression. The current research is focused on three classes of genes that may be associated, initially, with the multistage process of murine liver tumorigenesis: (1) cytochrome P-450 genes involved in metabolic activation of chemical carcinogens; (2) the known oncogenes, myc, ras and SV40 large T antigen, and (3) the developmental expression of a family of genes that contain sequences homologous to the Drosophilia homeobox. At present the work has involved setting up the transgenic mouse system.

PHS 6040 (Rev. 1/84)

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

Su-yun Chung	Sr. Staff Fellow	LEC	NCI
Snorri S. Thorgeirsson	Chief	LEC	NCI
Miriam Falzon	Visiting Fellow	LEC	NCI
Nancy Sanderson	Chemist	LEC	NCI

Objectives:

New developments in the past few years have made it possible to generate transgenic mice which carry introduced foreign genes in their germline genome. The technique involves microinjecting cloned DNA fragments into the pronucleus of a fertilized egg. A number of laboratories have reported that the introduced genes integrated into the host genome are stably transmitted to future generations following Mendelian inheritance. Many of the introduced genes are expressed in a tissue specific manner and the patterns of gene expression are transmitted to the offspring in some lines of transgenic animals. The overall objective of this study is to exploit the transgenic mouse system to introduce natural or manipulated gene sequences into the germline of an animal and to alter its phenotype and genetic background. This provides us a new way to investigate tissue specific and developmental stage specific regulation of gene expression. Furthermore, the interaction of the introduced gene and its biological effects on the host animal can be monitored throughout normal and malignant development from embryogenesis to adulthood. The current proposal focuses on three classes of genes that may be intimately associated with the multistage process of tumorigenesis in liver. Experiments on these classes of genes are described separately.

(1) We will introduce the rat cytochrome P-450C gene which is inducible by methylcholanthrene into the transgenic mice. Tissue specific gene expression, altered patterns of gene expression and their biological consequences in chemical carcinogenesis will be examined.

(2) We will introduce the known oncogenes, myc, vas, and SV40 large T antigen, under the control of a liver specific promoter system into the transgenic mice. The potential role in a multistep process and its effect on liver tumor development of each oncogene will be investigated.

(3) We will monitor the developmental expression of a family of genes that contain sequences homologous to the Drosophila homeobox in normal and transgenic mice. The ultimate goal is to look for an interplay among genes regulating development and genes affecting oncogenesis.

Methods Employed:

(1) Recombinant plasmid constructions and other recombinant DNA techniques.

(2) Embryo manipulations: fertilized eggs are obtained from one day pregnant females. The recombinant DNA fragments are microinjected into the pronucleus

of the fertilized egg. The injected mouse embryos are implanted into a pseudo-pregnant foster mother for further development. Under the current methodology about 10-30% of the injected embryos will survive and develop to term and about 30% of the newborns will carry the introduced gene in the germline.

(3) Germline integration: The positive animal and state of the introduced genes can be identified by Southern blot hybridization.

(4) Gene expression and gene products: RNA transcription in different tissues will be assayed by Northern blot hybridization or by S1 nuclease protection analysis. The presence of a gene product can be determined by the protein transfer method or by cytological immunofluorescence staining using antibody as a probe. For early stage embryos, the in situ hybridization method will be used.

(5) Histology and cell lines: Histological thin sections can be prepared from preneoplastic and tumor tissue and will be examined for pathological changes. Permanent cell lines can be established from tumor tissue for further investigation.

Major Findings:

The current experimental work has involved setting up the transgenic mouse system.

Significance to Biomedical Research and the Program of the Institute:

The transgenic mouse system offers a unique approach to study developmental regulation of genes that are potentially involved in the neoplastic process. Consequently, results obtained in this experimental system should provide valuable information on factors controlling the expression of these genes and possibly provide a basis for ultimately controlling the neoplastic process.

Proposed Course:

Continue as outlined under Objectives.

Publications:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05453-01 LEC

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Chemical Carcinogenesis; Transformation with Viral Oncogenes; Tumor Progression

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	Snorri S. Thorgeirsson	Chief	LEC	NCI
Others:	Ryuichi Konno	Visiting Fellow	LEC	NCI
	Ritva P. Evarts	Veterinary Medical Officer	LEC	NCI
	Su-yun Chung	Senior Staff Fellow	LEC	NCI
	Gordon L. Hager	Head, Hormone Action & Oncogenesis Section	LEC	NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Experimental Carcinogenesis

SECTION

Chemical Carcinogenesis Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.4	1.4	0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The main objective of this project is to compare the initiated state in the neoplastic process that is brought about by either chemical carcinogens or retroviral associated oncogenes and to determine the phenotypic traits that characterize the initiation caused by these different carcinogenic agents. We are employing rat liver and established normal rat hepatocyte cell lines as models in these studies. The research is currently focused on: 1) transfecting rat liver cells with molecular chimeras of MMTV-v-ras, and other retroviral associated oncogenes, that can be driven by glucocorticoid hormones and is known to transform 3T3 mouse fibroblasts; and 2) determine if morphologic transformation of the transfected hepatocytes is realized upon activation of the v-ras gene by dexamethasone. The results obtained so far include: 1) Rat hepatocyte cell lines derived from neonatal Fischer rats (FNRL-cells) were successfully transfected with pCneo10 plasmid, conferring G418 resistance. The transfection efficiency of the FNRL cells was about 25% of that observed in 3T3 cells. 2) Co-transfection of the FNRL cells with the neo-containing plasmid and the MMTV-v-ras chimera has resulted in isolation of several G418 resistant colonies that are presently being characterized.

PHS 6040 (Rev. 1/84)

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

Snorri S. Thorgeirsson	Chief	LEC	NCI
Ryuichi Konno	Visiting Fellow	LEC	NCI
Ritva P. Evarts	Veterinary Medical Officer	LEC	NCI
Su-yun Chung	Senior Staff Fellow	LEC	NCI
Gordon L. Hager	Head, Hormone Action & Oncogenesis Section	LEC	NCI

Objectives:

The main objectives of this project are to compare the initiated state in the neoplastic process that is brought about by either (1) chemical carcinogens or (2) retroviral associated oncogenes, and to determine the phenotypic traits that characterize the initiation caused by these different carcinogenic agents. We then plan to examine the in vivo progression of these initiated cells when subjected to a variety of promotional stimuli (phenobarbital, TCDD, etc.). We will employ the rat liver and established normal rat hepatocyte cell line as a model for these studies.

The research is currently focused on: (1) transfecting normal rat hepatocyte (established cell line) with molecular chimeras of MMTV-v-ras and other retroviral-associated oncogenes that can be driven by glucocorticoid hormones and is known to transform 3T3 mouse fibroblasts; (2) determine if morphologic transformation of the transfected hepatocytes is realized upon activation of the v-ras gene by dexamethasone; (3) characterize the cytomorphological and cytochemical changes, should transformation occur, and contrast these changes with those observed with chemically-initiated hepatocytes; (4) examine the response of these virally transformed cells to known promoters of chemically-induced hepatocarcinogenesis in both anchorage dependent and independent conditions, growth inhibitors and growth factors known to act upon rat hepatocytes, and (5) transplant the virally transformed hepatocytes into isogenic hosts (liver and anterior chamber of the eye) and characterize the growth and progression toward primary tumor formation under stimuli of glucocorticoid and/or liver tumor promoters.

Methods Employed:

Methods used in these studies include: tissue culture techniques, radioisotopic measurements, enzyme assays, histochemical and immunohistochemical methods, and recombinant DNA techniques.

Major Findings:

We have used a normal hepatocyte cell line derived from neonatal Fischer rats (FNRL) for transfection with plasmid DNA. The transfection method of Huang et al. (Cell 27: 245, 1981) with slight modifications was employed in these experiments. The results obtained so far include: (1) The FNRL cells were transfected with pCneo10 plasmid conferring resistance to G418 (neomycin). The number of G418 resistant colonies per 100 ng of the pCneo10 plasmid was

6 for the FNRL cells, whereas 22 G418 resistant colonies per 100 ng of the DNA were observed in the 3T3 cells; (2) cotransfection of the FNRL cells with the neo-containing plasmid and MMTV-v-ras chimera, and selection with G418 resistant colonies. These colonies are currently being characterized.

Significance to Biomedical Research and the Program of the Institute:

Our studies are aimed at identifying and characterizing both cellular and genetic factors that are important in initiation and progression of chemically and virally induced neoplasia. Information obtained from these studies may therefore provide better understanding of the cellular and genetic targets that are involved in the evolution of the neoplastic process caused by these two types of carcinogenic agents.

Proposed Course:

Continue as outlined under Objectives.

Publications:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01CP05454-01 LEC

PERIOD COVERED
October 1, 1984 to September 30, 1985

TITLE OF PROJECT (*80 characters or less. Title must fit on one line between the borders.*)
Chemical Transformation of Human Lymphoblastoid Cell Lines

PRINCIPAL INVESTIGATOR (*List other professional personnel below the Principal Investigator.*) (*Name, title, laboratory, and institute affiliation*)

PI:	Snorri S. Thorgeirsson	Chief	LEC	NCI
Others:	Dana Kessler	Microbiologist	LEC	NCI
	Carole A. Heilman	Senior Staff Fellow	LEC	NCI
	Jeff Cossman	Sr. Assistant Surgeon	CP	NCI

COOPERATING UNITS (*if any*)

None

LAB/BRANCH
Laboratory of Experimental Carcinogenesis

SECTION
Chemical Carcinogenesis Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.2	0.4	0.8

CHECK APPROPRIATE BOX(ES)
☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (*Use standard unreduced type. Do not exceed the space provided.*)
The objective of this project is to characterize the nature of chemically induced transition from a benign hyperproliferative to a malignant state in Epstein-Barr virus (EBV) immortalized human lymphocytes. Treatment with N-acetoxy-2-acetyl-aminofluorene (N-OAc-AAF), a potent frameshift mutagen, induced conversion of the EBV immortalized lymphocytes into high grade "immunoblastic lymphomas" on injection into athymic mice, whereas injection of the untreated, original cells did not. The tumor cells were all of the B cell lineage as determined by the presence of surface immunoglobulins and antigens detected by B cell specific antibodies to B1 and B4, and the absence of the T cell specific markers, 3A1 and LEU-1. The N-OAc-AAF-induced tumor lines displayed abnormal diploid to tetraploid karyotypes. The fewest chromosomal rearrangement, excluding tetraploidy, observed in these chemically induced lymphomas involved a deletion in chromosome 6 and additions on both 16 and 4. Neither major rearrangements nor amplifications were found for K-ras, H-ras, N-ras, c-myc, Blym and c-myb in these tumor lines. In order to determine the transforming genetic element(s) in our chemically induced lymphoma cells, we have transfected high molecular weight DNA derived from both the N-OAc-AAF treated CB23 (A23-15) and the original CB23 cell lines into NIH3T3 and have screened the resulting foci for the presence of oncogenes, including EBV fragments. The first round of transfection with DNA isolated from A23-15 cells gave rise to typical multilayered, well-defined foci. No foci were seen following transfection with DNA from the original untreated CB23 cells. Southern blot analysis of genomic DNAs derived from these transfected foci revealed the presence of human DNA as noted by Alu specific repeat sequences, yet none of the above-mentioned oncogenes were found in any of the transfected cells.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

Snorri S. Thorgeirsson	Chief	LEC	NCI
Dana Kessler	Microbiologist	LEC	NCI
Carole A. Heilman	Senior Staff Fellow	LEC	NCI
Jeff Cossman	Sr. Assistant Surgeon	CP	NCI

Objectives:

A considerable body of data from both animal experiments and epidemiologic studies provide strong evidence that cancer induction requires a minimum of two stages or events for the undirectional transition from a normal to a transformed state. A model for naturally occurring human cancer has been proposed in which the first event in the neoplastic process leads to an improperly controlled proliferation of cells (i.e., initiated cells), while the second event results in the commitment of the cells to develop into a clinically apparent cancer (JNCI 66: 1037-1052, 1981).

The objective of this project is to examine the nature of this second event (i.e., the transition from initiation to malignant transformation) in the context of chemically induced neoplasia. We have employed the Epstein-Barr virus (EBV), immortalized human cord blood lymphocytes (CB) and a human peripheral blood lympocyte culture derived from an EBV positive patient with infectious mononuleosis (Eckert) as the cell population that has sustained the "first" event in the neoplastic process. These cells have acquired the capacity to grow indefinitely in vitro, and may therefore represent the "initiated" cell. Since the natural history, including cytogenetic changes as well as oncogene activation, of the EBV carrying African form of human Burkitt's lymphoma has been extensively documented, it provides an excellent source of comparison as to the possible etiological role of chemical carcinogens in this disease as well as to the nature of the transition from initiated to transformed cells.

Methods Employed:

Methods used in these studies include: tissue culture techniques; differentiated centrifugation and chromatographic techniques; radioisotopic measurements using tritium, carbon-14, phosphorus-32 and iodine-125; cell surface analysis by fluorescence activated cell sorting; chromosomal analysis; and recombinant and molecular technology including DNA and RNA preparations, Northern and Southern blotting, and nucleic acid hybridization.

Major Findings:

1) In Vitro Transformation and Transplantation. Early passage CB and Eckert cells were exposed to the chemicals for 24 hours, washed, replated and viability determined. Viabilities ranged from 86% to 24% with the Eckert line showing the greatest sensitivity to N-acetoxy-2-acetylaminofluorene N-OAc-AAF. After 20 population doublings, cells were injected subcutaneously into 7 to 12 gram athymic

mice. Within two weeks palpable tumors (7 mm^3) were found in mice injected with CB23 cells treated with 15 μg/ml of N-OAc-AAF. By three weeks, all high dose (15 μg/ml) N-OAc-AAF treated cultures, as well as 5 μg/ml treated Eckert cell line, caused similar tumor growth in vivo. One untreated CB23 innoculum gave rise to a palpable tumor within two weeks, but had thoroughly regressed by four weeks. Contrary to this, the treated lines gave rise to aggressively growing tumors, reaching sizes of 15 mm^3 in four weeks. One DMSO treated CB34 control gave rise to a histologically identical tumor when compared to those which developed in the N-OAc-AAF treated lines. The MNNG treated lines did not give rise to any cells capable of tumor formation in vivo.

At four weeks growth, the tumors were aseptically excised from the animals. Half of the tumor was taken for histology and half was recultured. After 5 population doublings, the cultured primary tumor lines were reinjected into athymic mice. Tumor formation occurred within one week this time and the same tumor type was generated. The N-OAc-AAF induced tumors were histologically classified as high grade "immunoblastic" lymphomas showing thickened nuclear membrane, round oval nuclei and densely packed cells with prominent nucleoli.

2) Cell Surface Characteristics and Karyotypes of the Cell Lines of N-OAc-AAF Induced Tumors. The cell cultures derived from the tumors were assessed for cell surface characteristics by fluorescent activated cell sorting analysis. The cells were all of the B lineage as determined by the presence of surface immunoglobulins and antigens detected by antibodies to B_1 and B_4, and the absence of $3A_1$ and LEU-1 reactivity in those cultures tested (T-cell associated). Apparently, clonal selection of the cells has occurred in vivo as noted by the change in predominant light chain production in the untreated parental lines, CB23 and Eckert, and the treated tumor counterparts. The simultaneous presence of B_1, B_4 and surface immunoglobulins suggests that these cells are of an intermediate maturity, between pre-B cells and plasma cells. Three of the tumor lines (A23-15, A34-15, and D34) have begun to produce surface IgD, suggesting that immunoglobulin maturation and DNA rearrangements may be occurring.

Karyotyping of the CB and Eckert lines, after 30 population doublings in vitro, revealed an essentially normal diploid to slight aneuploid chromosomal complement, with CB23 displaying a modal chromosome number of 47 (80% of all cells). On the other hand the N-OAc-AAF induced tumor lines displayed an abnormal diploid to tetraploid karyotype. The abnormal diploid karyotype found in one of the N-OAc-AAF induced tumors ($A23\text{-}15_1$) was characterized by deletion in chromosome 6, and additions in chromosomes 16 and 4. The other two N-OAc-AAF induced tumors, as well as the spontaneously occurring tumor, showed tetraploid karyotypes.

3) Oncogene Expression of Rearrangement in N-OAc-AAF Induced Tumors. Since the karyotype revealed N-OAc-AAF induced chromosomal rearrangements, the possibility that oncogene rearrangement might have occurred was investigated. Southern blot analysis of total restricted genomic DNA derived from both the untreated parental lines and the N-OAc-AAF induced tumor lines was assessed for major rearrangements in oncogenes associated with human lymphomas (c-myc and Blym) or with fragile sites on chromosome 6, c-myb. Similar analysis was also performed for the ras family members, K-ras, H-ras, and N-ras, since these genes are commonly associated with human tumors, and one of these genes (i.e., c-K-ras-1) has been localized on human chromosome 6 (6p11-12, N. Popescu, personal communication). Under these

conditions, no differences in arrangements were noted, nor was amplification of any of these oncogenes found as illustrated by the Southern blot analysis to N-ras. Total poly(A)+ RNA was isolated from both sets of cell lines and analyzed by Northern blots. Again no increase in expression of any of the above oncogenes was observed.

EBV copy number was also determined by slot blot analysis and contrary to other reports concerning chemical carcinogen treatment of EBV immortalized CB lines, no increase in EBV viral DNA was observed. Conversely, a slight decrease in copy number, of approximately 10 copies per genome, was noted in all the chemically transformed lines when compared to their nontransformed counterparts.

4) Transforming Genetic Element(s) in N-OAc-AAF Induced Lymphoma. In order to determine the transforming genetic element(s) in our chemically induced lymphoma cells, we have transfected high molecular weight DNA derived from both the N-OAc-AAF treated CB23 (A23-15) and the original CB23 cell lines into NIH3T3 and have screened the resulting foci for the presence of oncogenes, including EBV fragments.

The first round of transfection with DNA isolated from A23-15 cells gave rise to typical multilayered, well-defined foci. No foci were seen following transfection with DNA from the original untreated CB23 cells. Southern blot analysis of genomic DNAs derived from these transfected foci revealed the presence of human DNA as noted by Alu specific repeat sequences, yet none of the above-mentioned oncogenes were found in any of the transfected cells.

Significance to Biomedical Research and the Program of the Institute:

Our studies are aimed at characterizing the chemically induced transition from benign hyperproliferative to the malignant transformed state using EBV immortalized human lymphocytes as our model system. The information obtained from these studies may provide a better definition of the cellular and genetic targets involved in the transition from benign to malignant state and provide clues to cancer cause and possibly help in formulating an effective cancer prevention program.

Proposed Course:

Continue the course outlined under Objectives and Major Findings.

Publications:

Kessler, D., Heilman, C. A., Cossman, J., Maguire, R. T. and Thorgeirsson, S. S.: Transformation of EBV immortalized human B cells by chemical carcinogens. Cancer Res. (In Press)

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05455-01 LEC

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Molecular Cloning of Genes Associated with Chemical Hepatocarcinogenesis

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P.I.	Carole A. Heilman	Senior Staff Fellow	LEC	NCI
Others:	Snorri S. Thorgeirsson	Chief	LEC	NCI
	Irene B. Glowinski	Staff Fellow	LEC	NCI
	Brian Huber	Staff Fellow (PRAT)	LEC	NCI
	Dana Kessler	Microbiologist	LEC	NCI
	Su-yun Chung	Senior Staff Fellow	LEC	NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Experimental Carcinogenesis

SECTION

Chemical Carcinogenesis Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.5	1.3	0.2

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The object of this project is to isolate and identify genes which are associated with neoplastic transformation in the rat liver system. This project will be in joint affiliation with other members of the laboratory who have isolated, or are in the process of isolating, proteins which are characteristic of the neoplastic state. To this end we have (1) constructed cDNA libraries in gamma-glutamyl transpeptidase II to both normal and neoplastic rat liver, (2) constructed a genomic library to the Fischer rat genome using EMBL vector system, and (3) are in the process of subcloning and sequencing a cDNA clone previously found to be associated with regenerating rat liver. We have also isolated a genomic clone for the widely used liver tumor marker, gamma-glutamyl transpeptidase, and gene expression studies with this clone are in progress.

PHS 6040 (Rev 1/84)

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged in the Project:

Carole A. Heilman	Senior Staff Fellow	LEC	NCI
Snorri S. Thorgeirsson	Chief	LEC	NCI
Irene B. Glowinski	Staff Fellow	LEC	NCI
Brian Huber	Staff Fellow (PRAT)	LEC	NCI
Dana Kessler	Microbiologist	LEC	NCI
Su-yun Chung	Senior STaff Fellow	LEC	NCI

Objectives:

The object of this project is to isolate and identify genes which are associated with neoplastic transformation in the rat liver system by employing molecular biological techniques. Our aim is to identify and study the modulation of these genes during tumor development using the rat liver system as the experimental model.

Methods Employed:

Methods used in these studies include: recombinant and molecular technology including DNA and RNA preparation, Northern and Southern blotting, construction of cDNA and genomic libraries and nucleic acid hybridization.

Major Findings:

Isolation of cDNA Recombinant Clones Which are Up-Regulated in Actively Proliferating Rat Liver. A cDNA library was constructed from poly(A) RNA obtained from 18-hour posthepatectomized (70%) Fischer rats. From this cDNA library a clone bank of 6,000 colonies was isolated and screened by the method of differential hybridization to identify clones that corresponded to genes which were specifically "turned on" 18 hours after 70% hepatectomy as compared to "resting" adult liver. No qualitative changes were seen in gene expression; however, four unique clones were isolated, containing cDNA inserts which varied from 600-2,000 base pairs (b.p.) which were up-regulated in regenerating liver. One of the clones has been initially characterized. It consists of approximately 1,080 b.p. and by slot blot analysis to poly(A) RNA has been shown to be three times more abundant in 18-hour posthepatectomized liver than sham-operated controls. In addition, Northern blot analysis has shown that this clone is also up-regulated in the livers of rats chronically fed phenobarbital or injected with 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), two potent tumor promoter regimens. In contrast, this gene was down-regulated in frank liver tumors, preneoplastic nodules produced by the Solt-Farber method, in the rat hepatoma line, 7777, and liver which has completely regenerated. Although this gene has not yet been identified, certain possibilities have been eliminated. Southern blot analysis of the cDNA insert has shown no homology to rat P-450 enzymes, inducible by 3-methylcholanthrene or phenobarbital, ornithine decarboxylase, or PRO-2, a promoter related gene in the

epidermis. We are presently recloning this gene in M13 for nucleic acid sequence analysis and will further characterize this gene following isolation in a recently constructed rat genomic library.

Construction of cDNA libraries in λ-gt II. The cDNA library of choice which we are presently utilizing is the λ-gt II system. Briefly, this system offers several long-range advantages. The cDNA is linked to two arms of the λ gt II phage and packaged as a recombinant phage. The phage is infected onto appropriate ndicator bacteria and grown in the presence of the chromogenic substrate X gal. Wild type phage are identified by their ability to utilize X gal following derepression of their lac operon (IPTG) and will be blue. Recombinant phage which has interrupted their lac operon by insertion of the cDNA will be unable to utilize X gal and will be identified as clear plaques. The efficiency of this system in our hands is 1×10^7 recombinants/μg cDNA. We presently have two libraries to (a) normal rat liver and (b) Solt-Farber induced rat hepatoma containing between 50,000-75,000 recombinants. In addition to high yields, the bacteria will also make a fusion protein, containing at least part of the protein coded by the inserted cDNA, thus allowing identification of clones by monoclonal antibodies. We hope to apply this technique to antibodies raised against unique proteins identified and isolated using the two-dimensional gel system. In addition, other members of the laboratory are purifying and sequencing proteins of interest from which synthetic DNA probes can be made and used to identify clones in these libraries. Finally, a genomic library was constructed to the Fischer rat genome using the EMBL vector system which can also be used for further analysis of the genes of interest.

Cloning of Rat γ-Glutamyl Transpeptidase (GGT) Gene. The heavy and light subunits of rat kidney GGT were purified to homogeneity after papain treatment. N-Terminal amino acid sequencing of both fragments was performed and the first 32 and 29 amino acids from the heavy and light subunits, respectively, were identified. Six amino acid sections of both the heavy and light GGT subunits were chosen for synthesis of the corresponding oligonucleotide series (17-mers). Due to the total amount of "wobble" in the light subunit, two different oligomers were synthesized differing only at position 9.

A rat genomic library made in Charon 4A (a gift of Dr. T. Sargent, Laboratory of Molecular Genetics, NICHD) was plated on NZCYM-agar plates using bacterial strain LE392 as host (16,000-20,000 plaques/plate). Plaques were transferred to nitrocellulose in triplicate, denatured in 0.5 M NaOH, 1.5 M NaCl and neutralized in 0.5 M Tris HCl, pH 7.5, 1.5 M NaCl. Nitrocellulose filters containing DNA were baked at 80° in vacuo for 2 hours.

Synthetic oligonucleotides were end-labeled using ^{32}P-γ-ATP and T4-polynucleotide kinase and used to screen the library for GGT. Nitrocellulose filters were prehybridized at 65° for 2 hours in a solution containing 6X NET (0.9 M NaCl, 0.09 M TrisHCl, pH 7.5, 0.006 M EDTA), 10X Denhardt's (0.2% albumin, 0.2% Ficol, 0.2% polyvinylpyrrolidine), 0.1% SDS and denatured herring sperm DNA (50 μg/ml) as carrier. For screening with the heavy chain probe, filters were hybridized at 42 for 4 hours in the same solution as for prehybridization without carrier and containing end-labeled probe ($\sim 1 \times 10^7$ cpm/ml; specific activity $\sim 1 \times 10^9$ cpm/μg). For screening with the light subunit probes, the procedure was exactly the same except that hybridization was carried out at 37° due to the

lower Tm (melting temperature) of these DNAs. After hybridization, filters were washed in 6X SSC at 0° for 15 min. 4 times, then in 6X SSC at room temperature for 15 min. 2 times, and then in 6X SSC at 37° for 15 min. After drying, the filters were autoradiographed overnight a -70° using intensifying screens and XAR-5 film.

Out of 480,000 plaques screened, 1 plaque was positive for both the heavy and light subunit probes. The phages from the positive plaque (#64) were replated on NZCYM-agar plates (at a low density), transferred in triplicate to nitrocellulose filters and rescreened with the heavy and light subunit probes, as described above. On the secondary round of screening, all plaques derived from the original positive plaque #64 were again positive for probes made from both subunits.

Large cultures of phage from plaque #64 were grown and the phage purified using CsCl gradients. The DNA was then extracted and used for further characterization of this clone.

GGT-DNA was cleaved using a series of restriction enzymes, run on agarose gels, and stained with ethidium bromide. EcoR1 cleavage of the DNA revealed four bands, two representing the arms of vector DNA and two representing the insert containing an internal EcoR1 site. The two bands of the insert migrated with molecular weights of approximately 5.8 and 7.2 kb. Cleavage with other enzymes gave a series of bands of varying sizes. DNA fragments were then transferred from the gels to nitrocellulose and baked for two hours at 80° in vacuo. Screening of the restricted DNAs revealed that an EcoR1 fragment (~ 7.2 kb), an EcoRV fragment (~ 2 kb), and a Hind III fragment (~ 1.2 kb) hybridized to the heavy and light subunit probes. The smaller EcoRV and Hind III fragments are being subcloned into pBR322 for further mapping, sequencing and gene expression studies.

Significance to Biomedical Research and the Program of the Institute:

Our studies are aimed at identifying and characterizing both the cellular and genetic factors important in chemically-induced and spontaneous neoplasia. The information obtained from these studies could provide a basis for a better definition of the factors involved in cancer cause and may help in formulating an effective cancer prevention program.

Proposed Course:

Continue the course outlined under Objectives and Major Findings.

Publications:

None

ANNUAL REPORT OF

THE LABORATORY OF EXPERIMENTAL PATHOLOGY

NATIONAL CANCER INSTITUTE

October 1, 1984 through September 30, 1985

The Laboratory of Experimental Pathology plans, develops and implements research on the experimental pathology of carcinogenesis, especially concerned with the induction of neoplasia by chemical and physical factors in epithelial tissues, including: (1) development, characterization and evaluation of experimental pathology models of human cancer, such as cancers of the respiratory tract, by in vivo and in vitro carcinogenesis methods; (2) development and characterization of tissue culture systems for quantitative study of the effects of carcinogens alone or in combinations; and (3) research on mechanisms of carcinogenesis correlating different levels of biological organization, from whole organisms (human and animal), organs and tissues, to the cellular, subcellular and molecular levels.

General research objectives:

The main program of investigations in the Laboratory of Experimental Pathology (LEP) is concerned with two correlated problems: (1) the pathogenesis of chemically induced neoplastic disease, particularly in lining epithelia, which are the tissues of origin of most human cancers, studied at sequential levels of biological organization, ranging from human tissues and animal models to organ and cell cultures, and to the biochemical and molecular levels; and (2) the interactions resulting from concurrent effects of different factors in multifactorial carcinogenesis mechanisms, including the role of carcinogens, promoters, oncogenes, growth factors, cellular mediators (e.g., reactive oxygen) and certain types of tissue injury.

Sequential series of biological models linking molecular, cellular and organ levels:

There is a fundamental need to relate the process of carcinogenesis to the specific characteristics of the tissues and cells from which the induced tumors originate and their conditions of exposure. Carcinogenesis induced by chemical and physical agents is the result of molecular interactions leading to pathologic responses that are typical of the different tissues and cells of origin. Cancer pathogenesis is characterized by a wide variety of pathologic response patterns in humans as well as in experimental animals. In order to correlate mechanisms of carcinogenesis, investigated at the cellular and molecular levels, with the corresponding events in animal and human tissues and organs, it is important to connect these different levels of observation.

In order to define the connections among pathogenetic models of carcinogenesis, the effects of carcinogens are studied in a series of biological systems related to each other in a step-by-step sequence. Such systems include molecular targets, cultured cell systems, organized tissues in culture and in vivo, and finally organs and whole organisms, including not only models of animal pathology but also human pathology. Such an approach requires the development of a range of

biological models related to human cancer pathology and particularly to those epithelial target tissues from which most of the major forms of human cancer originate. A great deal of progress has occurred in this direction in the past two decades and experimental animal models have been established, by chemical induction, for most of the major types of human cancer; for many of these models adequate culture systems have been developed for the target tissues and cells, including both animal and human target cells; pathogenetic mechanisms have been clarified, considerably, through major advances in experimental pathology, cell biology, molecular biology and biochemistry. Work in the LEP has contributed substantially to this progress. The current LEP program represents a logical sequence to these advances.

Cellular models and mechanisms:

Major emphasis has been given in the LEP to the study of human and animal epithelial tissues and cells in culture and in vivo. Current studies are focussed on cell culture systems for rodent and human epithelial cell systems related to human cancer pathology or corresponding to in vivo animal models well known for their response to carcinogens, such as the respiratory tract in rats and hamsters, the mouse skin, the human prostate and bladder. Emphasis is given to the use of serum-free, possibly chemically defined media. In vivo studies of carcinogenesis by chemical and physical agents focus on the induction mechanisms of respiratory tract tumors.

The following main epithelial systems are currently used in the LEP for studies on mechanisms of carcinogenesis:

(A) Respiratory epithelia - The hamster respiratory carcinogenesis model (Saffiotti, U., et al., Cancer Res. 28: 104-124, 1968) has become well established as closely resembling the pathogenesis of human bronchogenic carcinoma and continues to be used for in vivo studies of cell differentiation and tumor induction by various carcinogens and cofactors. Organ culture systems were established for hamster respiratory epithelia, and cell culture systems in serum-free media recently have been investigated for these target cells. The rat offers respiratory carcinogenesis models that differ considerably from those in hamsters. Rat respiratory epithelial culture systems have been well established by work in other laboratories which has led recently to the development of cell culture and transformation systems for rat respiratory epithelial cells; further developments in this laboratory have now established methods for the culture and transformation of these cells in serum-free media.

Extensive in vivo studies of respiratory tract carcinogenesis in both hamsters and rats are under way in the Laboratory. Induced tumors provide a source for the culture and characterization of malignant cells and for studies on the activation of oncogenes.

(B) Epidermal keratinocytes - The mouse epidermal carcinogenesis model has been widely studied in vivo for decades for its response to full carcinogens and/or promoting agents, such as 12-O-tetradecanoylphorbol-13-acetate (TPA). Previous work in the LEP led to the development of primary culture methods for mouse keratinocytes in conventional media for the study of differentiation and transformation (Yuspa, S., et al., Transplant. Proc. 12: suppl. 1, 114-122, 1980) and to the establishment of mouse epidermal cell lines (JB-6 and clonal sublines) transformable by promoting agents (Colburn, N. H., et al., Teratogen. Carcinogen. Mutagen.

1: 87, 1980). Currently, serum-free and nearly chemically defined media have been developed for the culture of mouse keratinocytes and for studies on transformation by carcinogens. The cellular response to hormones, growth factors and inhibitors is quantitatively determined in clonal assays. Studies on transformation methods are underway.

In collaboration with the Cell Biology Section, Laboratory of Viral Carcinogenesis, studies on the JB-6 clonal sublines that are either sensitive or resistant to promoter-induced transformation showed that promoter-sensitivity can be transmitted by DNA transfection from the sensitive to the resistant clones. Using this assay, two genes, pro-1 and pro-2, were isolated and then cloned and sequenced; the functional significance of the sequences is currently under study. The role of these genes is being studied in the JB-6 system and also in the primary keratinocyte culture system developed in the LEP. The JB-6 cells were also used in studies on mechanisms of promotion, e.g., to demonstrate the important role of reactive oxygen.

(C) Prostate epithelia - A normal prostate epithelial cell line and a corresponding prostate carcinoma cell line, established previously, are used (Lechner, J. F., J. Natl. Cancer Inst. 60: 797-801, 1978; Kaighn, M. E., et al., Urology 17: 16-23, 1979). A study is underway on the identification of transforming genes present in the carcinoma line and their role in the transformation of the normal epithelial line. Collaborative studies have continued on the characterization of the metastasizing variants.

(D) Other Epithelia - Human urothelium (bladder and ureter epithelium) was cultured using serum-free media, and the conditions for optimal response to growth and transformation are under investigation. The corresponding animal models for chemically induced bladder carcinogenesis were previously studied in the hamster and rat and may provide useful culture counterparts for in vivo/in vitro studies; the carcinogenic activity of several chemical carcinogens on the human bladder has been well established in occupational and environmental studies. Liver cell lines were used in bioenergetic studies and other liver cell preparations were used for some studies on carcinogen binding.

(E) Non-epithelial Cell Systems - Selected studies are conducted on non-epithelial cell systems for which appropriate culture conditions are established. (1) BALB/3T3 clone A31-1-1, a mouse embryo cell line, is used for quantitative analysis of carcinogen-induced neoplastic transformation, mutation and DNA damage/repair mechanisms; and for studies on chemically induced activation of transforming genes. (2) Chinese hamster ovary (CHO) cells are used for quantitative studies on mutation and DNA damage/repair; (3) NIH/3T3 cell lines are used for transfection assays of DNA-mediated transforming activity.

Development of chemically defined culture conditions for studies of differentiation and transformation in epithelial systems:

Methods for chemically induced neoplastic transformation of epithelial cells in culture started to develop in the last decade, but these methods need to be further extended and more rigorously defined from a quantitative point of view. As new and better defined culture conditions are established for target epithelial cell systems, their response to carcinogens needs to be correlated with the mechanisms

of neoplastic transformation investigated at the molecular level. Some of the epithelial systems described above (e.g., mouse keratinocytes, respiratory epithelia) have comparable patterns of response to specific treatments and culture conditions that can lead, on the one hand, to terminal differentiation, senescence and cell death and, on the other hand, to progressive cell growth, anchorage independence and neoplastic transformation. An important condition for studies on these mechanisms is the ability to grow the target epithelia in serum-free, possibly chemically defined culture media, replacing serum with selected additions of hormones and growth factors at optimal concentrations. Factors that control either continuous cell growth or the induction of senescence and terminal differentiation --and the escape from senescence of transformed cell populations--are under investigation.

Elimination of serum from the culture media in several cells systems has not only disposed of a source of uncontrolled biological variables from batch to batch, but also made it possible to analyze the specific role of individual growth factors and inhibitors in the control of cell growth and transformation.

Mechanisms of concurrent or sequential effects of different carcinogens and cofactors in multifactorial carcinogenesis:

A. Quantitative studies of transformation, mutation, toxicity and DNA damage/repair induced by carcinogens in selected cell systems. Concurrent induction of these different biological responses in selected cell systems has been quantitatively investigated under various experimental protocols that revealed patterns of dissociation of the responses. Of particular interest was the finding of a marked temporal dissociation between mutation and transformation responses obtained by varying the exposure durations to alkylating agents in BALB/3T3 cells, resulting in an early maximal induction of ouabain resistance (oua^r) mutations and single strand breaks, whereas a much longer exposure duration was required for the maximal induction of transformation and of cytotoxicity. Mechanism studies are in progress to analyze the molecular basis for this new phenomenon.

The establishment of quantitative assays for mouse keratinocytes and rat tracheal epithelial cells has made it possible to investigate spontaneous and induced transformation mechanisms quantitatively also in these epithelial systems.

B. Identification and characterization of genes involved in carcinogenesis. A new molecular biology unit has been equipped and activated in the LEP. Investigation of transforming genes has been extended to several cell systems under study in the Laboratory. Current work is devoted to: (a) the identification of three separate transforming DNAs obtained from BALB/3T3 cells transformed by benzo[a]-pyrene (BP) and having different sensitivity to restriction enzymes; (b) the identification of oncogenes in a human prostatic carcinoma cell line; (c) the role of single or multiple oncogenes transfected in normal and preneoplastic rat tracheal epithelial cells; and (d) the functional analysis of the pro-1 and pro-2 genes that were previously cloned and sequenced, and that control promoter-dependent transformation.

C. Investigation of biochemical mechanisms in carcinogenesis models. The following areas of investigation have been pursued with biochemical and biophysical methods relevant to carcinogenesis models. (a) The role of reactive oxygen and

free radicals has been shown to be involved in the response of epithelial mouse skin cells to tumor promotion, and is going to be further studied in relation to macrophage-mediated stimulation of pulmonary epithelia in silica-treated lungs. (b) The role of protein kinase C and heat-stress proteins in the mechanisms of tumor promotion was analyzed. (c) Bioenergetic pathways and the characteristics of the lactic dehydrogenase (LDH) system were studied in non-transformed and in neoplastic rat liver cells; preliminary findings suggest that cells acquiring preneoplastic properties by repeated passages show a sharp increase in LDH activity before undergoing morphological changes: the possible significance of this biochemical step as an early marker of transformation will be pursued. (d) Studies of benzo[a]pyrene metabolism and DNA binding were conducted comparatively in various segments of the hamster respiratory tract in vivo and in vitro. (e) The binding of carcinogens to specific DNA regions was investigated by methods that distinguish binding to DNAase I-hypersensitive regions from the remainder of DNA: high binding levels were found in such DNA regions in preliminary experiments. (f) Methods recently developed in the Laboratory of Pathology, DCBD, NCI, for the detection of collagenase IV activity have been selected for studies on the timing of induction of this enzyme during neoplastic transformation. Activation of this enzyme, which digests basal membrane collagen, is needed for cell invasiveness. These studies are designed to test whether this enzyme can be used as a marker for the acquisition of established malignant transformation in various cell types.

In vivo studies on multifactorial mechanisms of respiratory carcinogenesis:

In vivo studies are mostly devoted to respiratory carcinogenesis, continuing a long line of research in this field by the present investigators. Two strains of hamsters (inbred and outbred) and a strain of rats are used both for long-term experiments on induced tumor pathology and for serial sacrifice experiments designed to study the stages of tumor pathogenesis, the cells of origin and the concomitant pathology following treatment protocols with different carcinogens and cofactors. Current emphasis centers on the role of combined treatments with topical and systemic chemical carcinogens, with or without physical factors such as microtrauma and inorganic particulates, and/or other toxic chemicals. The kinetics of the proliferative response of the target epithelium is studied to characterize the cells of origin of the hyperplastic, metaplastic and neoplastic reactions of the respiratory epithelium. Different segments of the respiratory tract are studied for their different responses to various experimental conditions, including the nasal epithelium, the trachea, the bronchi and the peripheral airways. Most of these studies, initiated in the new animal facilities assigned to LEP in the past two years, are still under way.

A new research approach has been developed on the basis of previous and current findings linking the granulomatous fibrogenic reaction induced by crystalline silica in rat lungs with an early and sustained hyperplastic proliferation of the peripheral airway epithelia and with the eventual induction of pulmonary carcinomas. The hamster provides a negative model, lacking both the fibrogenic and the carcinogenic responses to silica. The working hypothesis has been proposed that the intense granulomatous reaction induced by silica (macrophages, fibroblasts, lymphocytes, plasmacells, mastcells, and polymorphonuclear leukocytes), previously studied in detail, generates a continuous release of cellular mediators of inflammation which act to stimulate the adjacent respiratory epithelium. These mediators include reactive oxygen (already known to be involved in epithelial cell stimulation

and promotion), as well as many cytokines (e.g., interleukin-1), enzymes, and several other factors, that have not been adequately studied for their effects on target epithelia. This new research approach has far-reaching implications for a fresh re-evaluation--by modern methods and in specific terms--of the relationship between certain chronic inflammatory reactions and carcinogenesis. The silica model, in vivo and in vitro, offers an excellent opportunity to clarify these mechanisms.

In summary, LEP research is addressed to the pathogenesis of epithelial neoplasia, through the elucidation of possibly complementary mechanisms that induce neoplastic transformation when concurrently or sequentially activated, studied in animal models correlated with the main epithelial tissues of origin of human cancers in vivo, in the corresponding cell culture models, maintained by optimally defined culture conditions, and finally at the biochemical and molecular level.

OFFICE OF THE CHIEF

(1) Provides overall scientific direction and administrative coordination to the Laboratory's intramural research program and its supporting resources; (2) participates in research projects in all components of the Laboratory and provides collaborative research coordination of staff activities and resources; (3) conducts bibliographic research and data analysis; and (4) conducts research on carcinogenesis mechanisms and quantitative studies on the interactive effects of combined exposures to different carcinogens and cofactors, using in vivo and in vitro systems established in the Laboratory.

Investigators assigned to this Office conduct basic research on mechanisms of neoplastic transformation of cells in culture, on biochemical mechanisms and on the molecular characterization of oncogenes involved in chemical carcinogenesis and tumor promotion.

Projects are under way in the following areas:

Quantitative studies on the concurrent induction of cytotoxicity, DNA damage and repair, mutation and transformation. In previous studies, the mouse embryo cell line, BALB/3T3 clone A31-1-1, was found susceptible to neoplastic transformation induced by a number of carcinogens with different metabolic pathways, including polycyclic aromatic hydrocarbons, aflatoxin, aromatic amines, nitrosamides and arsenic. A mutation assay for oubain resistance (oua^r) was also established. Alkaline elution was used to study DNA damage and repair. Studies on repair kinetics of individual DNA adducts were conducted by HPLC.

Dissociation patterns between different end points in their response to carcinogen treatment were previously demonstrated in BALB/3T3 cells in the following test conditions: (1) Synchronization of cells in the exponential growth phase and treatment with N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) for 30 min, at various points during the cell cycle showed cell cycle dependence for mutation and independence for transformation. (2) Split-doses of MNNG, compared with single-dose treatments, showed no evidence for recovery from sublethal damage. Repair of DNA damage, as measured by alkaline elution, was demonstrated by split-dose treatment, but cytotoxicity, mutation and transformation were not affected by dose splitting. (3) Exposures for different periods of time to media containing MNNG showed that the exposure periods required for maximal induction differed for different end-points: short for DNA damage measured by alkaline elution and for oua^r mutations

(30-60 min), and long for cytotoxicity and for neoplastic transformation frequencies (120-240 min).

Duration of exposure was studied with the ethylating agent, N-ethylnitrosourea (ENU), with a half-life of about 12 min, and showed the same dissociation phenomenon, but shifted to shorter exposure times (<5 min for DNA damage and mutation, >45 min for cytotoxicity and for transformation).

The molecular mechanisms involved in this newly observed temporal dissociation phenomenon were further investigated. The DNA repair kinetics determined by alkaline elution showed a rapid initial repair rate, both for MNNG and for ENU. This mechanism cannot, however, be affecting transformation frequencies which continue to increase until late exposure times. The repair of alkylated DNA adducts was measured by HPLC using established procedures. After 30 and 60 min post-treatment incubations, BALB/3T3 cells were found capable of rapid repair of O^6-ethylguanine and moderate repair of N3-ethyladenine, but showed no removal of N7-G, O^4-T and phosphotriester adducts. Comparable studies were performed with the Chinese hamster ovary (CHO) cells, which were known to be unable to repair O^6-alkylguanine adducts. This property was confirmed both in short- and in long-term repair assays. Surprisingly, CHO cells showed the same early maximal induction of oua^r-mutations and single strand breaks observed in BALB/3T3 cells. In contrast, 6-thioguanine resistance ($6\text{-}TG^r$) mutations (detectable in the CHO cell line) showed slower induction kinetics, compatible with a theoretical curve based on a linear response to ENU as exponentially decaying in time. $6\text{-}TG^r$ mutations, but not oua^r mutations, may thus be correlated with O^6-alkylation of guanine in this system. Further analysis of repair kinetics by more sensitive methods are planned in order to elucidate the molecular basis for the observed divergent temporal responses to mutations and to transformation in different cell lines.

Identification, characterization and cloning of oncogenes in chemically transformed cells or related systems. The mechanisms whereby different genes control carcinogenesis induced by chemical and physical agents are investigated in the molecular biology unit recently established and equipped within the Laboratory.

These studies are directly correlated with the characterization of cellular systems under study in the Laboratory. Methods employed in these investigations include: DNA transfection followed by selection of specific phenotypes; gene cloning using the sib selection protocol and library screening with specific probes; restriction mapping; subcloning and sequencing of cloned genes; Southern and Northern transfer techniques; treatment of nuclei with DNAase I to locate DNA-carcinogen adducts and to probe the chromosomal structure of new transforming genes.

The following studies have been conducted:

(1) Identification of oncogenes activated by carcinogens in BALB/3T3 cells. It was previously reported that DNA from 3 cell lines obtained from different benzo-[a]pyrene-transformed BALB/3T3 foci showed different sensitivities to digestion with restriction endonucleases. The transforming DNAs were identified by repeated transfections in NIH/3T3 cells. Hybridization studies showed no evidence of involvement of the ras gene. Genomic libraries have now been constructed from each of these three DNAs with different activity, using Charon 4A phage, and the transforming genes are being isolated by the sib selection procedure. These

studies will clarify whether a single carcinogen can affect different transforming genes in the same cell line and may possibly identify new transforming genes.

(2) Cloning of transforming gene(s) from human prostate carcinoma (PC-3 cells). The well-characterized human prostatic carcinoma cell line (PC-3) and its non-transformed counterpart cell line (NP-2s), obtained from normal human prostate epithelial cells, were previously isolated by Kaighn and coworkers (See: Tissue Culture Section) and are being used to investigate the nature of the transforming genes present in PC-3 cells. PC-3 DNA was transfected into the normal NP-2s epithelial cells and the treated cells were cultured for selection of altered clones showing anchorage independence or escape from senescence. Further analysis of such clones is under way. In a parallel study, PC-3 DNA was transfected into NIH/3T3 cells and transforming activity was detected. A genomic library is being constructed from DNA of secondary transformants in order to isolate and characterize the transforming human gene(s).

(3) Structural analysis of pro genes. Two mouse genes were previously identified in a collaborative project with the Cell Biology Section, LVC, from promoter-sensitive (P^+) clones from the mouse keratinocyte JB-6 cell lines. These genes induce the P^+ phenotype when transfected in promoter-resistant (P^-) clones, that are tested for anchorage-independent growth in the presence of the promoting agent TPA. These two genes (pro-1 and pro-2) were cloned and sequenced. The sequences are now being investigated for their structural characteristics and functional significance. These two genes were found to be different from each other, to have no homology with known oncogenes and to present unusual structural features. Pro-1 is relatively small (approximately 1 kb) and its sequence appears as a fusion sequence assembled from two types of middle repetitive elements, the BAM5 and the Alu-type B1 repeat, joined by an apparently unique sequence of G4bp. The promoter elements are in an ordered spatial arrangement typical of polII promoters; they are present downstream to translation terminator codons. The open reading frame is contiguous with no introns and predicts a product of 65 amino acids (MW 7,100 daltons) with a highly unusual composition. The pro-2 genomic segment is larger (3.8 kb) and mostly formed by a unique sequence, except for a small middle repeat element that is unrelated to known mouse repeats.

Investigation of biochemical mechanisms of carcinogenesis.

Several biochemical approaches were used for studies releated to models of chemically induced carcinogenesis in epithelial cells.

(1) Role of reactive oxygen and free radicals in carcinogenesis. The induction of anchorage-independent growth in JB-6 mouse epidermal cells treated with the promoting agent, TPA, was used as an assay to investigate the role of oxygen radical generators and their inhibitors. Distinct roles were found for different species of reactive oxygen free radicals. Inhibition of their effects on promotion was found to vary for various reactive oxygen eliminators; superoxide dismutase was particularly effective in blocking TPA-induced transformation if added to the cells prior to or within 4 hours of TPA exposure. The theoretical pathways related to free-radical mechanisms in carcinogenesis were analyzed. The role of reactive oxygen, produced by stimulated macrophages and other cells involved in granulomatous reactions, is now under study for its effects on pulmonary epithelia in the silica model.

(2) The role of protein kinase C and of heat-stress proteins in the mechanisms of tumor promotion. Protein kinase-C (PK-C) activity and substrates were found to remain similar when studied in JB-6 mouse keratinocyte cell lines with different susceptibility to transformation induced by the tumor promoter, TPA. The substrates were categorized according to cation conditions favoring their phosphorylation. A heat-shock protein, pp80, previously reported to be deficient in tumor cell lines but increasing in response to TPA treatment in untransformed JB-6 cell lines, was found not to be a PK-C substrate. Studies on temperature-sensitive effects of gamma-interferon are under way.

(3) Bioenergetic pathways in transformed epithelial cells.

This research activity was moved to newly designed laboratory space in LEP, and renovations were completed by January 1985. In this new facility, cell culture conditions were reestablished, and biochemical/biophysical base line conditions and experimental sensitivity standards were determined for all instruments. The instruments include those for protein separations, HPLC (with computer), gel electrophoresis, isoelectric focussing, and the Immobiline system; kinetic analysis with water-clear enzyme solutions and with light-scattering preparations such as whole cells and mitochondria; and single-beam spectrophotometer for measurements of steady-state activity.

These studies pertain to the development of aerobic glycolysis in cancer cells, using current biochemical methods to identify modifications in lactate dehydrogenase (LDH, E.C. 1.1.1.27), cytoskeletal complexes and mitochondria.

The results of studies with rat liver cells showed that the steady-state activity of LDH has a ratio of 1:4 between control and chemically-transformed neoplastic cell lines. Specific isozymes associated with either control or neoplastic liver cell lines were determined. Different ratios of LDH-4 and -5 were identified when control liver cell lines in passages up to 21 were compared with neoplastic cell lines. As the passage number increased, control cells showed alterations in the ratio of enzyme activity relative to neoplastic cells, reaching 1:1, and in the ratio of LDH-4 and -5, which also shifted to 1:1. These LDH studies will be extended to investigate whether such LDH protein changes may serve as an early indicator of preneoplastic events in control cells. Isozymes characteristic of both epithelial and fibroblastic lines remain associated with the original tissue type in both the control cell lines and the transformed neoplastic cell lines. In the case of both epithelial and fibroblastic tissue types, a more basic form of LDH appears with either chemical transformation or viral transformation to neoplastic cell lines.

Modifications in cytoskeletal complex formations and in mitochondria are under investigation in the control and chemically-transformed rat liver epithelial cell lines to expand previous observations of ultrastructural modifications.

(4) Carcinogen metabolism and binding. Studies on the retention, distribution, metabolism and binding of carcinogens in target tissues and cells have been continued using both in vivo and in vitro model systems. Current experiments are devoted to the analysis of benzo[a]pyrene metabolism and DNA binding in various segments of the respiratory tract related to selective carcinogenic responses in the hamster and the rat models. Detailed findings are reported in their context under the Respiratory Carcinogenesis Section.

(5) Study of the role of DNAase I hypersensitive regions in carcinogenesis. The question whether carcinogens bind preferentially to selected regions of DNA was addressed by investigating the relative binding in the regions that are rapidly attacked by DNAase I. Preliminary studies in vivo and in vitro showed increased relative binding of carcinogens in these DNA regions, particularly in experiments measuring aflatoxin B_1 binding in isolated rat liver nuclei. Southern blot analysis of liver DNA revealed v-Ha-ras homologous sequences that were DNAase I hypersensitive, but no detectable rearrangement or amplification of this oncogene was found following treatment with carcinogens.

RESPIRATORY CARCINOGENESIS SECTION

(1) Conducts research on the pathogenesis of cancers in the respiratory tract and on their induction by carcinogens, alone or in combinations, using animal models closely related to human pathology and corresponding in vitro systems; (2) investigates the carcinogenic effects of chemical and physical agents on the respiratory tract, their quantitative aspects and their pathogenetic mechanisms; (3) studies mechanisms of cell differentiation and carcinogenesis in respiratory and related epithelia; and (4) provides pathology expertise, resources and collaboration to other components of the Laboratory in the study of epithelial carcinogenesis.

The research activities of this Section are devoted to the characterization of respiratory carcinogenesis responses in appropriate model systems in vivo and in vitro and to the elucidation of mechanisms of epithelial carcinogenesis by chemical and physical factors, alone or in combinations. The programs are closely correlated with those of the other LEP components to which this Section also provides pathology research expertise for in vivo animal carcinogenesis studies.

The hamster respiratory carcinogenesis model (Saffiotti, U., et al., Cancer Res. 28: 104-124, 1968) is used both for long-term and short-term studies on the combined effects of different factors and for the development of the corresponding epithelial organ culture and cell culture models for different segments of the respiratory tract in collaboration with the Tissue Culture Section. Two recently established colonies of Syrian golden hamsters (inbred strain 15:16/EHS:CR and non-inbred strain Syrian /CG.FOD) are used. This hamster model was previously extensively studied and shown to be closely similar to its human counterpart in its differentiation and pathogenesis; it represents, therefore, a model of choice for studies on mechanisms of induction of bronchogenic carcinoma.

Studies are continuing on cellular characterization in the different segments of the respiratory tract following intratracheal instillations of inorganic particulates and administration of different topical or systemic carcinogens, alone or in combinations. Histologic, histochemical, ultrastructural, immunochemical and autoradiographic techniques are used for the characterization of the segmental response of the respiratory epithelium to different carcinogens and their combinations.

Biochemical studies:

Studies were continued (in collaboration with the Office of the Chief) on the metabolic pathways and DNA binding of benzo[a]pyrene (BP) in the trachea, bronchi and peripheral lung tissues. In vivo binding of BP to the different segments of

the respiratory tract was determined at serial time points after a single intratracheal dose of BP or after a terminal dose preceded by 11 other weekly doses. Binding of the same test dose of BP was higher to tissues from hamsters receiving 12 weekly doses than a single dose: the ratio of the two groups reached about 5:1 for bronchi and for lung and 14:1 for the trachea. The overall DNA binding, measured as pmoles of BP per mg of DNA, consistently showed the relationship: trachea>bronchus>lung. Different time course patterns of binding were found in the tracheas after single or multiple doses; the single dose treatment showed that a binding maximum was reached by 24 hrs and maintained unchanged through 168 hrs, whereas the 12 dose group showed a peak at 24 hrs followed by a sharp decline after 96 hrs. In contrast, bronchi and lungs showed maxima at 24 hrs and no further declines for both treatment protocols. These findings are being correlated with the histological studies of epithelial reparative hyperplasia and sloughing off of outer epithelial layers observed following microtraumas induced by cannulation. In vitro studies on BP metabolism were conducted on hamster tracheal and bronchial explants after 20 hrs incubation in serum-free medium with 5mM BP incorporated into phosphatidyl choline liposomes. BP metabolites were analyzed and characterized by HPLC. The trachea and the bronchi produced different metabolite profiles. The trachea produced more total metabolites than the bronchi, both as total organ and on a per mg wet weight basis. The more active procarcinogenic metabolites were also formed in higher proportions in the trachea than in the bronchi. Similar experiments performed on tissue explants from hamsters that had been pretreated in vivo with a course of repeated BP instillations showed a significant, but transient, increase in BP metabolism in these target tissues of respiratory carcinogenesis.

Age as a susceptibility factor to respiratory carcinogenesis by diethylnitrosamine (DEN):

Experimental work was nearly completed and the results are being analyzed for a long-term study comparing respiratory tumor induction in hamsters treated with a course of systemic subcutaneous administrations of DEN, starting at one day, 4 weeks or 8 weeks of age. The one-day-old hamsters were found much more sensitive to respiratory carcinogenesis by DEN, as expected, but the patterns of tumor responses showed interesting variations. The incidence of malignant neoplasms was proportionally higher in hamsters treated at young age, whereas the incidence of induced tracheal papillomas (a typical response to DNA) was similar at all age groups. The morphology and cellular origin of the various types of induced tumors are under study.

Respiratory carcinogenesis in hamsters induced by benzo[a]pyrene (BP) combined with modifying factors:

BP-induced carcinogenesis in the hamster model is under investigation in a series of studies, using the established protocol of intratracheal administration of BP absorbed on ferric oxide particles. The carcinogenic response is being characterized in long-term studies in the inbred strain of hamsters 15:16/EHS:CR recently established at FCRF. Long-term studies are under way to determine whether 3-methylindole (3-MI), a cigarette smoke component with systemic toxicity for Clara cells and alveolar type I cells, alters the carcinogenic response induced by the BP/ferric oxide protocol. Preliminary results brought out an unexpected finding: hamsters that received BP/ferric oxide intratracheally in combination with intraperitoneal injections of 3MI in DMSO or just DMSO showed a significantly higher

incidence and earlier onset of tumors in different segments of the respiratory tract in comparison with the groups treated only with BP/ferric oxide. It was surprising, however, to find that no significant difference is so far apparent between the enhancement produced by 3MI in DMSO and that due to just DMSO, a finding that suggests that DMSO may function as an important modifier of respiratory carcinogenesis in this model.

A large in vivo long-term study, now nearing completion, is conducted in collaboration with the Department of Pathology, University of Maryland, to investigate the role of epithelial damage and proliferative response due to mechanical injury, inorganic particulates or saline, in the carcinogenic response to different carcinogens in the hamster system. The induced pathology is evaluated by histological, histochemical and electron microscopic methods to identify the cellular types involved in these reactions and the kinetics of their response.

Relationships of the granulomatous fibrogenic reaction to silica and epithelial carcinogenesis in the lungs:

A major new research approach has been developed on the basis of past studies and of recent findings on the pulmonary reactions to crystalline silica dusts. Evidence has been reported from several laboratories that quartz dust induces not only progressive granulomatous fibrogenic reactions (silicosis), but also carcinomas arising from the pulmonary epithelia in rats. In hamsters, the same dust preparations failed to induce either fibrosis or tumors (Goldsmith, Shy and Winn, Eds., Silica, Silicosis and Cancer, Praeger Publ., Philadelphia, in press). The association of epithelial proliferation and granulomatous reaction has been studied in this Laboratory in histologic material from previously conducted experiments in rats and from new experimental groups of F-344 rats. A marked proliferation and hyperplasia of the epithelium of the peripheral airways was observed in areas of silica-induced granulomatous reaction as early as a few days after intratracheal administration of reactive silica dusts (hydrofluoric acid washed quartz; tridymite). The cellular composition and evolution of silica-induced granulomatous reactions in rats had been previously extensively studied (Saffiotti, U., Med. Lavoro 53: 5-10, 1962) and it is currently being reinvestigated with additional techniques. The main cell types involved in the granulomatous reaction to silica in the lung include: macrophages (which are killed by the insoluble crystalline silica particles, inducing a cycling reaction of macrophage necrosis, recruitment and phagocytosis), lymphocytes, plasmacells, mastcells, polymorphonuclear leukocytes and fibroblasts. Progressive deposition of collagen and immunoglobulins form the typical silicotic nodules, still surrounded by a granulomatous reaction. The epithelial proliferation was found to take place in airways surrounded by an adjacent granulomatous reaction. The cells of the granulomatous reaction have been the object of extensive immunopathology studies in the last decade and a number of biologically active mediators are known to be released by these cells, including: reactive oxygen species, various cytokines (e.g., interleukin-1), complement components, various enzymes, and the components of the arachidonic acid cascade. It has been suggested as a working hypothesis (Saffiotti, U., in Goldsmith et al., op. cit.) that silica may not only damage epithelial cells directly but may also induce their continuous proliferation through intense and prolonged stimulation of granulomatous cells which release cell mediators capable of inducing damage, growth stimulation or both in the adjacent epithelia. The hamster model, negative for both fibrogenic and carcinogenic effects of silica is studied in parallel to the rat. Preliminary results show that toxicity of silica is very low in hamster

macrophages and high in rats. This new project will extend the in vivo investigation of the cellular reactions and carcinogenic responses of rats and hamsters to silica, with and without chemical carcinogens. In addition it will use methods for the culture and characterization of macrophages and other cells of the granulomatous response (in collaboration with the Laboratory of Molecular Immunoregulation, BRMP, DCT, NCI) as well as methods for the culture of epithelial cells developed in this Laboratory to investigate the interactions of granulomatous cell mediators on epithelial target cells. Preliminary experiments showed a dose-dependent inhibition of colony formation by a commercial interleukin-1 preparation in the rat tracheal epithelial cell culture assay used in the Laboratory.

In view of the role of reactive oxygen on epithelial cell transformation, demonstrated in another project in this Laboratory, additional studies have been started to investigate the role of reactive oxygen (produced by stimulated macrophages) as a mediator in silica-induced carcinogenesis, in vivo and in vitro.

TISSUE CULTURE SECTION

(1) Conducts research on cell culture systems for the characterization and quantitative study of neoplastic transformation induced by chemical and physical carcinogens; (2) develops and characterizes organ and cell culture systems for carcinogenesis studies, especially those derived from epithelia known for their susceptibility to carcinogens in vivo, such as the respiratory tract epithelium, (3) conducts research on mutagenesis, neoplastic transformation, differentiation, and on their expression mechanisms and relationships; and (4) provides expertise, resources and collaboration on tissue culture methods for the entire Laboratory.

The objective of the research program of the Tissue Culture Section is to understand how carcinogenic agents alter the control of growth and differentiation at both the cellular and molecular levels and how cells progress to neoplasia through a sequence of genomic and/or epigenetic alterations. Emphasis is placed on studies of epithelial cells derived from tissues representing major organ sites relevant to human cancer. Cell systems currently under study include mouse epidermal keratinocytes, rat and hamster respiratory epithelial cells and human prostatic epithelial cells. In addition, mouse embryo cell lines (BALB/3T3) and NIH/3T3 are used for studies on transformation mechanisms (See also: Office of the Chief).

Growth control in epithelial cells and its alteration in carcinogenesis:

Serum-free media have now been developed for both mouse keratinocytes (MK) and rat tracheal epithelial (RTE) cells. The keratinocyte medium, LEP-1, consists of Eagle's MEM without calcium, with non-essential amino acids and the following supplements: hydrocortisone, 0.5 uM; insulin, 5 ug/ml; phosphoethanolamine and ethanolamine, 50 uM each; transferrin, 5 ug/ml; epidermal growth factor, 5 ng/ml; bovine pituitary extract, 180 ug of protein/ml. The culture system is dependent for growth on bovine pituitary extract as the only remaining undefined supplement. LEP-1 supports sustained multiplication of mouse keratinocytes for 25 or more population doublings. A clonal growth assay was used to measure the activity of growth factors, hormones and other supplements. When the calcium concentration of the medium was raised to 1.0 mM or when 1 to 3% fetal bovine serum was added, the cells underwent terminal differentiation as confirmed by electron microscopy

and by immunostaining with antikeratin antibody. Serum factors were shown to have either stimulatory (albumin) or inhibitory activity (fetuin, crude platelet extract, TGF beta). This finding explains why keratinocytes cannot be carried beyond primary cultures in serum supplemented media. When albumin (100 ug/ml) was added to LEP-1, MK cells continued to multiply and are now at the 33rd passage. Chromosomal studies showed an altered karyotype by the 2nd or 3rd passage. By passage 17, most cells are near triploid or tetraploid with random loss or gain of individual chromosomes. This mouse keratinocyte system in serum-free medium is currently used in studies of chemically induced transformation and gene activation, using both secondary and late (17-33) passage cells.

A serum-free medium was also developed for rat tracheal epithelial (RTE) cell cultures consisting of Ham's F12 with 0.8 mM calcium, 15 mM HEPES buffer and 9 factors: epidermal growth factor 5 ng/ml, transferrin 5ug/ml, insulin 5 ug/ml, hydrocortisone 0.5 uM, ethanolamine 50 uM, phosphoethanolamine 50 uM, cholera toxin 1nM, bovine pituitary extract 1% (v/v), and bovine serum albumin 500 ug/ml. This serum-free cell culture system is currently being used in studies of RTE cell growth control, transformation, and differentiation. EG variants could also be induced by chemical carcinogens in serum-free culture.

Primary RTE cells formed colonies in this medium with an efficiency of 5%. RTE cells switched from serum-free medium to a serum-containing medium (F12, 5% serum, insulin, hydrocortisone) gradually stopped multiplying and underwent a process of squamous differentiation. This growth-inhibitory property made possible the use of serum-supplemented medium to select for preneoplastic enhanced growth (EG) variants of RTE cells. EG variants arose spontaneously in serum-free cultures of RTE cells. As the number of RTE cells per culture increased with time (5 to 23 days in culture), the frequency of EG variants per RTE colony also increased. In contrast, the rate at which EG variants appeared remained constant with time at approximately 6 x 10^{-6} variants/cell/generation.

The role of serum-derived factors in transformation:

The serum-free media systems, now available for normal epithelial cell systems under study in the Section, are being used to develop selective media for transformed cells. Transformed fibroblasts have long been known to have a reduced serum "requirement." In the case of at least some epithelial cells this situation is reversed; normal epidermal cells, tracheal epithelium and human urothelium terminally differentiate in the presence of serum, whereas the growth of their transformed tumorigenic counterparts is stimulated. Chemically-transformed mouse keratinocytes have been grown in serum-supplemented media (Yuspa, S. H., et al., Cancer Res. 40: 4694-4703, 1980). Thus, following exposure to a carcinogen and initial growth under serum-free conditions, the cells could be switched to serum to select for serum-resistant variants as has been done with RTE cells. Similarly, since normal MK cells were found to require bovine pituitary extract (BPE), a BPE-free medium could be used for selection of variants. This type of change in growth control could be one of the earliest manifestations of the multistage neoplastic process. This screening process could be used to identify the critical factors that distinguish normal from transformed cells.

Oncogenes and transformation:

Work in this Section is particularly concerned with epithelial cell systems in which both transformed cells and their normal counterparts can be grown in

culture. In such systems, the DNA of the transformed cells can be transfected into the appropriate normal cells and analyzed for its ability to induce neoplastic or preneoplastic properties for the identification of the responsible genes. As reported under the Office of the Chief, this approach was successful for the mouse keratinocyte cell line, JB-6, in providing a system for the identification of the promoter-related pro genes which are now being investigated for their functional role.

The hypothesis that differential responses of "preneoplastic" cell lines to carcinogens or oncogenic DNAs have a genetic basis is being tested in the RTE system. Preneoplastic RTE cell lines are treated with N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) or with cloned oncogene-containing DNAs from Harvey murine sarcoma virus (v-Ha-ras), polyoma virus, and MC29 virus (myc). Responses of the individual cell lines to these various treatments are compared by measuring the tumorigenicity of the treated lines in athymic nude mice. Three variants treated with a genomic clone of polyoma virus became tumorigenic, whereas only one of the three formed tumors after transfection of V-Ha-ras. The presence and expression of the V-Ha-ras gene is being examined. Strategies are planned for cloning RTE cell tumor genes. Transfections of normal RTE cells with cloned oncogene-containing DNAs are also described.

Transformation of human cells in culture has proven to be very difficult. The reasons for this problem are unknown but may be related to a number of "steps" required for the full expression of neoplasia in human cells, derived as they are from a species with a long life span and a long latent period for cancer in vivo. Thus, in order to compress a 20-30 year process in vivo into a short time frame in culture it may be necessary to "hit" the cells with a sequence of carcinogens and growth factors and to activate specific oncogenes. A normal human prostatic epithelial cell line (NP-2s) and its neoplastic counterpart (PC-3) derived from a bone metastasis of prostatic adenocarcinoma, were previously established (Kaighn, M.E., et al., Invest. Urol. 17: 16-23, 1979). The prostate is an important organ site for cancer in man, and prostatic cancer is the third leading cause of cancer deaths in males in the United States. Collaborative studies were initiated last year with J. F. Lechner of the Laboratory of Human Carcinogenesis to investigate the role of oncogenes in the human prostatic epithelial line, NP-2s, that had been "transformed" by SV40 virus (NP-2s/T2) but acquired neither the capacity for unlimited growth nor tumorigenicity in nude mice. In an effort to induce the cells to progress toward neoplasia, they were superinfected with Kirsten sarcoma virus or transfected with a plasmid vector carrying the EJ-ras oncogene. Both treatments resulted in new cell lines with extended life spans which contain the ras oncogene and secrete a transforming growth factor (TGF) for NRK cells. However, they have not become tumorigenic in nude mice. As an extension of this work, DNA from PC-3 is being transfected into the normal prostatic epithelial cells (NP-2s) by the calcium phosphate precipitation technique. Since NP-2s cells have a limited life span, escape from "senescence" or from growth limitation in culture will be used to assay for and isolate oncogene-altered lines.

A new study (in collaboration with M. I. Lerman, Office of the Chief) seeks to identify known or possibly new oncogenes in the established neoplastic cell line PC-3 by DNA transfection into NIH/3T3 cells. Transformed colonies have been isolated and expanded and secondary transfections are in progress. Further characterization of this transfecting activity will be carried out by established methods.

Studies on metastatic properties of cell lines:

The metastatic capability of the human prostatic adenocarcinoma cell line (PC-3) in nude mice has been investigated in collaboration with Dr. James M. Kozlowski (University of Chicago). The incidence of metastasis was increased by the isolation of variant sublines from secondary tumor deposits. For example, a PC-3 variant (PC-3-M) isolated from a lung metastasis produced 13 times the incidence of pulmonary metastasis as did the parental line after i.v. injection. The basis of this enhancement of metastatic capability in variant lines is unknown. The fact that alternate metastatic phenotypes can be selected may be correlated with different responses to growth factors, substrates and even activated or amplified oncogenes. Additional experiments with a PC-3-M line metastatic to the liver are being initiated.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01CP04491-09 LEP

PERIOD COVERED
October 1, 1984 through September 30, 1985

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)
Quantitative Studies on Concurrent Factors in Neoplastic Transformation

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	U. Saffiotti	Chief	LEP	NCI
Others:	M. E. Kaighn	Expert	LEP	NCI

COOPERATING UNITS (if any)
Laboratory of Toxicology, Istituto Superiore di Sanita', Rome, Italy
(M. Bignami, E. Dogliotti).

LAB/BRANCH
Laboratory of Experimental Pathology

SECTION

INSTITUTE AND LOCATION
NCI, NIH, Frederick, Maryland 21701

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.7	0.3	0.4

CHECK APPROPRIATE BOX(ES)
☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The BALB/3T3 clone A31-1-1 cell line was studied with the following assays: cytotoxicity, ouabain resistance (oua-r) mutations, morphological neoplastic transformation, DNA damage and repair as measured by alkaline elution and removal of alkylated DNA adducts as measured by HPLC. The relative levels of response for these biological end points were determined after treatment with the alkylating agents, N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) and N-ethylnitrosourea (ENU). A new dissociation phenomenon, reported last year, was confirmed and further investigated this year. Duration of the exposure to a medium containing a given initial concentration of ENU determined maximal responses that differed remarkably for different end-points. Short exposure duration times (5 min) were sufficient to induce maximal levels of oua-r mutations and of DNA single strand breaks (ssb), whereas the maximal induction of transformation as well as of cytotoxicity required 45-60 min. Additional studies of exposure time-dependent responses were conducted with ENU in Chinese hamster ovary (CHO) cells, for which both oua-r mutations and 6-thioguanine resistance (6-TG-r) mutations can be assayed. The induction kinetics of oua-r mutations and ssb in CHO cells showed early maximal induction analogous to that observed in the BALB/3T3 cells, whereas 6-TG-r mutations were induced at progressively higher rates in time, consistent with a theoretical curve based on a linear relationship to exponential decay of ENU in the medium. BALB/3T3 cells removed O6-ethylguanine rapidly and N3-ethyladenine to a lesser degree, whereas no removal within 60 min was detected for N7-ethylguanine, O4-ethylthymine and ethyl-phosphotriesters. The CHO cells, however, were not capable of repairing O6-ethylguanine lesions. Repair of O6-alkylguanine, therefore, is unlikely to explain the early maximal response of oua-r mutations and ssb. The temporal dissociation model is being further studied to clarify the basis for the long induction times needed for transformation.

PHS 6040 (Rev. 1/84)

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

U. Saffiotti	Chief	LEP NCI
M. E. Kaighn	Expert	LEP NCI

Objectives:

To study mammalian cell culture systems for concurrent induction of cytotoxicity, DNA damage and repair, mutagenicity and neoplastic transformation in order to define the interrelationships of these end points in response to multiple factors or to different experimental conditions; and to analyze the comparative mechanisms of such responses.

Methods Employed:

The BALB/3T3 clone A31-1-1 mouse embryo cell line was used under test conditions previously standardized in this laboratory for transformation assays with different carcinogens and for the induction of ouabain resistance (oua^r). Analogous studies were also conducted in Chinese hamster ovary (CHO) cells for oua^r mutations and 6-thioguanine-resistant ($6\text{-}TG^r$) mutations. DNA damage and repair were determined by alkaline elution analysis and removal of alkylated DNA adducts was measured by HPLC analysis.

Duration of exposure to alkylating agents was studied in time-course experiments for toxicity, mutation, transformation, DNA damage and repair. Two alkylating agents were used for treatments: N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) and N-ethylnitrosourea (ENU).

Major Findings:

Dissociation of measured end points was previously reported in BALB/3T3 cells under various experimental conditions.

(a) Split-dose treatments with MNNG with varying intervals between two equal split doses, compared with corresponding single doses, showed no recovery from sublethal damage and no significant effects on the frequencies of oua^r mutations or transformation, whereas repair of DNA damage detected by alkaline elution was significantly demonstrated by split-dose treatment with intervals from 1 to 5 hrs.

(b) Treatment of this cell line with MNNG for 30 min at various points during the cell cycle showed maximal induction of oua^r mutations throughout the S phase and low induction in the G_1 phase, but a constant level of transformation frequencies was found in G_1, early S and late S phases. These results show a dissociation of mutation from transformation in their cell cycle dependence in this cell line, which differs in this respect from the C3H 10T1/2 line.

(c) Duration of exposure to alkylating agents was found to be an important parameter that results in a marked temporal dissociation of different biological responses in both BALB/3T3 and CHO cells.

The effects of varying the duration of exposure to MNNG in BALB/3T3 cells were reported last year. The results showed that maximal induction of cytotoxicity was reached late (about 120 min), whereas maximal induction of oua^r mutations was reached early (30-60 min), as was the maximum level of single strand breaks (ssb). In contrast, the induction of morphological neoplastic transformation reached a maximum level very late (120-240 min). The half-life for MNNG in the cultures was about 68 min. Similar studies have now been completed with another alkylating agent, ENU (half-life of about 14 min). The same pattern of temporal dissociation of biological responses was found, but shifted to much earlier times. DNA damage and mutation reached their maxima already after 5-min exposures, while cytotoxicity and transformation reached their maxima only after 45-60 min, with a high transformation/mutation ratio.

DNA repair, as measured by rejoining of ssb in alkaline elution tests, showed a rapid repair rate (repair appears completed in 30-60 min). The observed DNA repair may, therefore, be considered as a mechanism involved in the early plateau for the induction of ssb and oua^r mutations, but it cannot be related to the continuing increase of transformation rates until 45-60 min of exposure. The repair of alkylated DNA adducts was determined by HPLC using the procedure of Beranek et al. (Carcinogenesis, 1:595-606, 1980) after 30 and 60 min post-treatment incubation. BALB/3T3 cells were found capable of rapid repair of O^6-ethylguanine and moderate repair of N3-ethyladenine but no removal of N7-ethylguanine, O^4-ethylthymine and of ethyl-phosphotriesters was detected.

Similar studies were conducted in CHO cells exposed to ENU for different periods of time. The CHO cells were known to be unable to repair O^6-alkylguanine lesions and this property was confirmed in short- and long-term repair tests. In spite of this marked difference in repair capacity of CHO cells as compared to BALB/3T3 cells, both cell types showed the same rapid response to maximal induction of oua^r mutations and ssb. In contrast, $6\text{-}TG^r$ mutations in CHO cells showed markedly different induction kinetics, with progressive increases for 45-60 min of exposure time, closely following a theoretical curve calculated on the basis of a linear response to ENU as exponentially decaying in time.

Significance to Biomedical Research and the Program of the Institute:

These studies are part of a long-term project designed to investigate the quantitative response of cellular systems to the concurrent induction of mutation and neoplastic transformation, as a basis for quantitative studies on the combined effects of different carcinogens and cofactors. The present findings support the hypothesis that different molecular mechanisms are involved in the induction of mutation, of transformation, and of DNA damage, as measured by selected methods. The temporal dissociation of exposure times required for maximal induction of mutation and transformation by both MNNG and ENU provides a new biological model for investigating the differences between mutation and transformation mechanisms, especially in relation to molecular target mechanisms.

Proposed Course:

Further analysis of the repair kinetics of different DNA adducts in this system; analysis of the factors involved in the continuing induction of transformation during exposure periods when the mutagenic response is already saturated, particularly by the use of treatment-conditioned media and of selective inhibitors of DNA repair enzymes.

Publications:

Bignami, M., Dogliotti, E., Benigni, R., Kaighn, M. E. and Saffiotti, U.: Split-dose exposure to N-methyl-N'-nitro-N-nitrosoguanidine in BALB/3T3 ClA31-1-1 cells: Evidence of DNA repair by alkaline elution without changes in cell survival, mutation and transformation rates. Mutat. Res. 145: 81-88, 1985.

Saffiotti, U.: Comparability of in vitro and in vivo systems for carcinogenesis evaluations in different species, tissues and cells. In Vouk, V., Butler, G. C., Hoel, D. G., and Peakall, D. B. (Eds.): Methods for Estimating Risks of Chemical Injuries: Human and Non-human Biota Ecosystems. Sussex, England, John Wiley & Sons, 1984, pp. 235-245.

Saffiotti, U., Bignami, M., Bertolero, F., Cortesi, E., Ficorella, C. and Kaighn, M. E.: Studies on chemically induced neoplastic transformation and mutation in the BALB/3T3 Cl A31-1-1 cell line, in relation to the quantitative evaluation of carcinogens. Toxicol. Pathol. 12: 383-390, 1984.

Saffiotti, U., Bignami, M. and Kaighn, M.E.: Parameters affecting the relationships among cytotoxic, genotoxic, mutational and transformational responses in BALB/3T3 cells. In Barrett, J. C. (Ed.): Cell Transformation Assays. Raven Press, New York. (In Press)

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP04493-07 LEP

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Bioenergetic Pathways in Chemically-Transformed Epithelial Cells

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI: A. E. Kaplan Research Chemist LEP NCI

COOPERATING UNITS *(if any)*

Laboratory of Applied Studies, Division of Computer Research and Technology, NIH, Bethesda, MD (B. Bunow); Department of Microbiology, Harvard Medical School, Boston, MA (H. Amos); Program Resources, Inc., Frederick, MD (R. L. Brown)

LAB/BRANCH

Laboratory of Experimental Pathology

SECTION

Office of the Chief

INSTITUTE AND LOCATION

NCI, NIH, Frederick, Maryland 21701

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.2	1.0	1.2

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The purpose of this project is to characterize the enzyme pathways which support aerobic glycolysis in neoplastic cells and tumors. Studies in progress are the following: (1) Characterization of protein modifications in lactate dehydrogenase in relationship to the increased secretion of lactic acid in the chemically transformed neoplastic cell. Control rat liver epithelial cells in culture show a constant ratio of activity and LDH-4 and -5 compared with corresponding chemically transformed cells up to passage 21, but LDH activity in the control cells increases as the passage number increases above 21 and the LDH-4 and -5 become equal in reactivity. As the passage number increases, LDH from control cells approaches but does not equal that of the neoplastic cells. Comparison of LDH isozyme from epithelial and fibroblastic lines show that each retains the characteristics of the parent cell even following neoplastic transformation. (2) Studies on changes in the ultrastructure of control and neoplastic cells related to cytoskeletal complexes of tubulin, cytokeratin, and actin are underway. (3) Analyses of spectrophotometric absorption components of mitochondria, and analysis of total heme content of control and chemically-transformed neoplastic cell lines are being performed. (4) The breakdown product of heme proteins is evaluated in relationship to reduced numbers of mitochondria and depleted structures in neoplastic cells.

PHS 6040 (Rev. 1/84)

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

A. E. Kaplan Research Chemist LEP NCI

Objectives:

The current objectives of this project are:

(1) To characterize modifications in lactate dehydrogenase (LDH E. C. 1.1.1.27):

(a) in a chemically-transformed neoplastic cell line which shows alterations in protein markers, gel electrophoresis, isoelectric focusing and kinetic behavior relative to lactic acid secretion in its control cells;

(b) in cells at different passages, since biochemical modifications in LDH were observed in control rat liver cells when passed above passage 21; and

(c) by comparing LDH from epithelial and fibroblastic cell lines.

(2) To extend previous observations of ultrastructural differences in cytoskeletal complexes of neoplastic cells compared with control cells.

(3) To analyze the heme content of control and neoplastic cells and the spectrophotometric absorbances characteristic of the mitochondria in an effort to identify the decrease in heme bound to mitochondria in the neoplastic cell.

(4) To reestablish cell culture procedures, and base line conditions and standardized biochemical and biophysical instruments after this project was moved, in January 1985, to new space in LEP.

Methods Employed:

1. Standard biochemical techniques used to extract and stabilize LDH from control and neoplastic cell lines of both epithelial and fibroblast origin. Identification of isozymes by gel electrophoresis; pI values by the isolectric focusing and Immobiline systems.

2. Use of fluorescent antibody techniques to identify tubulin, cytokeratin, and actin complexes in cells to determine differences in the aggregation patterns between control and neoplastic epithelial cells.

3. Heme analysis carried out by fluorescence spectroscopy, and mitochondrial studies carried out with DW-2c Double-Beam Spectrophotometer (SLM-AMINCO).

Major Findings:

1. Differences in LDH patterns were demonstrated under the following conditions:

a. The control epithelial cell line from 10-day old rat liver, TRL, is used normally up to passage 21 for biochemical comparisons with NMU-3, a transformant derived from rat liver cells by incubation with N-nitrosomethylurea. Under these conditions, the ratio of LDH activity extracted from control compared with transformed cells is 1:4. The ratio of isozymes in control cells is LDH-4:LDH-5 = 4:1, whereas the reverse is observed in the neoplastic cell line. The secretion of lactic acid into the medium, however, is an order of magnitude higher in the NMU-3 compared with control cells. Thus in some way, the altered properties of LDH contribute much more actively to lactic acid secretion than would be expected.

b. In TRL control cells cultured beyond passage 21, it has been considered that morphological changes occur as an early expression of spontaneous transformation. However, it has been observed that distinct alterations in LDH develop by passage 22, although no change in appearance was observed by light microscopy. Instead of a ratio of LDH activity of 1:4 for TRL: NMU-3 as seen below passage 21, the activity in TRL cells was found to increase after P 22, so that the ratio reaches 1:1 by passage 26. At the same time, in the TRL cells, the ratio of LDH-4:LDH-5 drops from 4:1 to 1:1. However, the TRL cells have never been observed to shift in isozyme ratio so as to reach the ratios of NMU-3 cells. These results with higher passage TRL cells suggest that the protein changes identified with LDH precede the more complex changes identified with morphology related to spontaneous transformation. Further studies will be needed to determine the relationship of these protein changes to cell aging and/or preneoplastic changes that would precede the expression of more complex morphological changes related to spontaneous transformation.

c. Although most experiments in this laboratory are addressed to the question of changes in aerobic glycolysis in chemically-transformed neoplastic epithelial cells, some studies were extended to fibroblastic lines. In comparing cell pairs from the two types of tissue, it is evident that differences in LDH persist and maintain their isozyme identification relative to the tissue of origin. This was observed even when a morphologically atypical control-type epithelial line was established. Here the isozyme pattern correlated with the tissue of origin of the cell, that is, liver epithelial. In both epithelial and fibroblastic cells which have undergone neoplastic transformation, LDH with a more basic pI appears; in fibroblasts this basic shift is far smaller than that of the neoplastic epithelial cells. Furthermore, the subsequent pathways through which ATP is utilized appears to be defective. This prevents normal flow of energy for the anabolic needs of the cell and results in a constant loss of potential energy from the host organism.

2. The ultrastructural differences between TRL and NMU-3 have been reported (A. E. Kaplan, M. K. Yamaguchi, T. S. Tralka, and C. H. Hanna, Exptl. Cell Res., 138 (1982) 251-260) and show that the chemically-transformed neoplastic cell line is reduced in membrane elaboration and altered in cytosol and nuclear structure. However, the external architecture of the NMU-3 cell is very similar to the control, TRL, cell line. For this reason, comparison of LDH extracts is currently being extended to compare tubulin, cytokeratin and actin complexes in the two cell lines to see if subtle differences can be identified

internally in the cytoskeletal complexes. The three cytoskeletal protein complexes are believed to interact so that actin connects with the plasma membrane peripherally, then with the cytokeratin, which in turn is associated with the highest molecular weight cytoskeleton protein, tubulin. All three make up an interconnecting, three-dimensional network within the cytosol. Final results with the TRL and NMU-3 cells are due soon.

3. Mitochondrial studies have begun with comparative analysis of the intracellular heme content of TRL and NMU-3. The reason for this approach is that ultrastructural results consistently indicated that the mitochondria in NMU-3 cells are very defective in structure with respect to the external membrane, as well as the cristae and the fragmented matrix. In addition, the mitochondria in NMU-3 cells are numerically reduced by 35% in comparison with TRL controls. Thus there may be a constant breakdown of heme into the cytosol. The final results of these analyses are due soon and will then be compared with the spectrophotometric absorptions of mitochondria to evaluate the heme content which is bound to the mitochondria compared with the total heme content in the cell to determine the free heme in the cytosol.

Significance to Biomedical Research and the Program of the Institute:

Current biochemical and morphological probes used to examine changes in LDH and in mitochondria will serve to identify modifications in the glycolytic and oxidative systems of the neoplastic cell. These methods were not available in earlier years for analysis of the sources of positive entropy in the cell. A constant loss of energy in the host organism leads to weight loss in the patient, even though it arises from a relatively limited tumor mass. The classic observations regarding aerobic glycolysis in cancer tissue, made by Warburg in 1924, have never been clarified with respect to specific enzyme changes because adequate experimental tools were not available. However, current methods permit more accurate reevaluation of enzyme modifications and opens opportunities for application to cancer tissues.

Proposed Course:

Further details of the protein changes in LDH with chemical transformation of neoplastic cells will be explored with the Immobiline System (LKB) to obtain more accurate pI values than were available with the IEF system. In addition, work will begin on the identification of the protein structures of the LDH tetramers by HPLC methods. The results of the Immobiline analyses will be useful in developing more accurate methods to separate the LDH proteins on HPLC. These methods will be applied to both epithelial and fibroblast preparations of LDH for comparison of modification in protein structures.

Studies with cytoskeletal complexes will be completed and will continue if current results lead to additional experiments.

Spectrophotometric analyses of mitochondria will begin when heme analyses are completed. In addition to the experiments described above, studies will begin with Prof. C. P. Lee, Scholar of the University, Wayne State University, who

is an expert in the development and application of fluorescent probes to identify mitochondrial membrane proteins which convert energy from ATP to anabolic forms of energy used by the cell.

Publications:

None.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05265-04 LEP

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Effects of Chemical Carcinogens on Transforming DNA Sequences and Expression

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	M. I. Lerman	Visiting Scientist	LEP	NCI
Others:	M. Bignami	Guest Researcher	LEP	NCI
	R. L. Norman	Sr. Staff Fellow	LEP	NCI
	U. Saffiotti	Chief	LEP	NCI
	M. E. Kaighn	Expert	LEP	NCI
	D. Y. Goldgaber	Visiting Associate	LCNSS	NINCDS
	N. H. Colburn	Chief, Cell Biology Sect.	LVC	NCI

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Experimental Pathology

SECTION

Office of the Chief

INSTITUTE AND LOCATION

NCI, NIH, Frederick, Maryland 21701

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.4	1.9	0.5

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The aim of this project is to identify, characterize, and clone those genes that drive the development of neoplasia and whose malignant potential results from changes caused by chemical carcinogens. (1) To identify transforming genes activated by benzo[a]pyrene (BP), DNA from three BALB/3T3 cell lines transformed by BP was analyzed by DNA transfer and focus formation in the NIH/3T3 system. All three tested lines showed transforming activity that differed from each other and from the ras oncogenes by restriction endonuclease sensitivity and MspI mapping. These possibly new transforming genes are now being cloned by the sib selection protocol from Charon 4A phage genomic libraries. Three protocol cycles of selections have been carried out and the positive pools show no presence of the ras oncogenes. (2) To explore the mechanisms by which chemical carcinogens may activate proto-oncogenes, the distribution of aflatoxin B1 (AFB) adducts on genes was analyzed using purified liver nuclei and microsomes in vitro. AFB adducts were preferentially located in DNAse I hypersensitive regions of the genome. (3) To elucidate the genetic events underlying the mechanisms of tumor promotion in mouse skin, novel genes termed pro 1 and pro 2, specifying sensitivity to induction of transformation by TPA in JB-6 cells, were cloned by sib selection from a size-selected DNA library of clonal cells sensitive to promotion. By restriction mapping, heteroduplex analyses and direct hybridization, the pro genes are different from and unrelated to oncogenes or other known genes. Both pro genes have been sequenced and their functions are being investigated. Human homologs of pro genes have been isolated from the human nasopharyngeal carcinoma cell line CNE2. (4) Using the NIH/ 3T3 focus assay, a transforming gene was detected in human prostate carcinoma cell line PC-3 and is being cloned; these cells apparently do not contain altered ras oncogens.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

M. I. Lerman	Visiting Scientist	LEP	NCI
M. Bignami	Guest Researcher	LEP	NCI
R. L. Norman	Sr. Staff Fellow	LEP	NCI
U. Saffiotti	Chief	LEP	NCI
M. E. Kaighn	Expert	LEP	NCI
D. Y. Goldgaber	Visiting Scientist	LCNSS	NINCDS
N. H. Colburn	Chief, Cell Biol. Sect.	LVC	NCI

Objectives:

The overall objective of this project is to identify, characterize, and clone cancer genes that drive the development of neoplasia and whose malignant potential results from changes caused by chemical carcinogens. The immediate objectives are: (a) to determine the molecular structure of the transforming genes activated in BALB/3T3 cells transformed by benzo[a]pyrene and to obtain information on the molecular lesions responsible for their activation, (b) to further characterize the structure of a novel class of genes (pro-genes) involved in tumor promotion.

(a) Oncogenes activated in chemically transformed animal and human cells:

DNA is generally considered to be the major target for the initiation of carcinogenesis with most known chemicals. The knowledge of oncogenes suggests that they may be the specific target genes of carcinogens and that the chromosomal structure of specific oncogenes in a given cell, as well as the nature of the chemical agent, may determine the molecular mechanism by which the genes become activated.

Two approaches are pursued:

(1) Transformation is induced with different chemical carcinogens in normal animal and human cells (primary cultures) and in immortalized cells (cell lines). The DNA of the transformed cells is analyzed for transforming activity by transfection back into the nontransformed parent cells and used as a source for the cloning of activated oncogenes by established and novel approaches. The molecular lesions leading to activation will be investigated by comparing the alleles from transformed and nontransformed cells at the DNA sequence level.

(2) Brief treatment of nuclei with low concentrations of DNAase I has been used to define DNAase I hypersensitive regions in the chromosome. These DNAase I hypersensitive regions, when mapped, occur in the putative regulatory regions of actively expressed genes. Similar studies employing carcinogen treatment in vivo or in vitro followed by DNAase I treatment of the nuclei should determine whether or not carcinogens preferentially bind to DNAase I hypersensitive regions. Labeled probes of known oncogenes will be hybridized to blots of genomic DNA to study the effects of carcinogen and DNAase I treatments at the gene level.

(b) Genes involved in tumor promotion:

The biology of tumor promotion is well defined especially in mouse skin; the genes responsible for the response to promoters are under investigation. Isolation of clonal preneoplastic cell lines responding to tumor promoters with an irreversible induction of the transformed phenotype indicated that tumor promoters directly affect the initiated cell and suggested that specific genes may be involved. Direct DNA-mediated gene transfer from promoter-sensitive to promoter-resistant clones of the JB-6 cells demonstrated the presence of such genes. In a joint effort with the Cell Biology Section, LVC (see Project #Z01CP05382-02 LVC), these genes, termed pro, were cloned and they are currently being analyzed for their structure and function at the DNA sequence level. The mechanisms of their activation by chemicals are also under study. Human homologs are being isolated and investigated. The characteristics of the corresponding normal proto-pro genes are being investigated using primary cultures of mouse keratinocytes developed in Project #Z01CP05276-04 LEP.

Methods Employed:

Gene cloning techniques using the sib selection protocol and library screening with specific probes. Calcium-phosphate DNA transfection followed by selection for immortal phenotypes and/or anchorage-independent growth. Restriction mapping, subcloning and sequencing of the cloned genes by the Maxam and Gilbert technique. Southern and Northern transfection techniques to analyze genomic organization and expression of the cloned genes. Preparation of nuclei, DNA and RNA. Treatment of nuclei with DNAse I to locate DNA-carcinogen adducts and probe the chromosomal structure of new transforming gene(s).

Major Findings:

Identification of carcinogen-activated oncogenes in BALB/3T3 cells: As previously reported, DNA was extracted from a series of 16 BALB/3T3 cell lines obtained from foci transformed by different chemical carcinogens or by U.V. light. Transfection into NIH/3T3 cells showed transforming activity for 7 of the 16 lines. In order to investigate whether the same or different transforming sequences were induced by the same carcinogen, the three most active lines transformed by benzo[a]pyrene (BP) were studied further after digestion with the restriction endonucleases, EcoRI, HindIII, BamHI and XbaI. Different patterns of sensitivity to endonuclease activity were obtained and confirmed for each of the three tested lines. This shows that different patterns of sensitivity can be induced by the same carcinogen in the same target cells.

In these cell lines, hybridization with probes containing Ha-ras, Ki-ras and N-ras oncogenes repeatedly failed to show any mutation, rearrangement or amplification of the ras gene, implying that apparently different new transforming genes are present in these cell lines. To clone these genes Charon 4A phage genomic libraries were constructed, and the transforming genes are being isolated by sib selection using the NIH/3T3 focus assay. So far, after three cycles of selection, positive pools were obtained containing approximately 700 different recombinant phages which were all negative with the ras oncogene probes.

Cloning of a Transforming Gene from Human Prostate Carcinoma (PC-3) Cells:

Role of DNAase I hypersensitive regions in carcinogenesis: Initial results of in vivo experiments with hamsters indicated that the formation of DNA-BP adducts reaches a maximum in the liver within 30 to 60 minutes after injection of [^{3}H] BP into the portal vein. Of these adducts, 30 to 40% appear to be in DNAase I hypersensitive regions as determined by counting the high molecular weight DNA isolated from liver nuclei from BP-treated hamsters after treatment of the nuclei in vitro with DNAase I, as previously reported.

Southern blots of genomic DNA from the livers of hamsters dosed in vivo with [^{3}H] BP revealed that v-Ha-*ras* homologous sequences were present in hamster liver and were DNAase I hypersensitive. This hypersensitivity was not affected by BP-treatment in vivo. Analysis also indicated that no detectable rearrangements or amplification of the v-Ha-*ras* homologous sequences had occurred in liver DNA following BP treatment. A similar lack of effect of BP and aflatoxin B_1 was observed on v-Ha-*ras* homologous sequences in rat liver. Additional studies employing other oncogene probes are planned.

To further investigate the role of DNAase I hypersensitive regions in carcinogenesis, an in vitro system has been developed. Rat liver nuclei isolated by conventional procedures were incubated at 30°C with [^{3}H] aflatoxin and microsomes in the absence of Mg^{+2}. The nuclei were reisolated, resuspended and treated with low concentrations of DNAase I for 5 minutes at 30°C. DNA was isolated by the usual procedures and aliquots counted.

Concentrations of DNAase I which do not appear to alter the average molecular weight distribution of the DNA (about 1 unit/mg of DNA) caused a 40% loss of label indicating that aflatoxin, a known liver carcinogen in the rat, binds preferentially to DNAase I hypersensitive regions of the genome of rat liver cells. Additional studies to establish the optimal conditions and time dependence of this phenomena are in progress. Complementary studies of DNA repair of these lesions as well as further *in vivo* studies are planned.

Structural analysis of pro genes:

Two mouse *pro* genes (pro 1 and pro 2) specifying sensitivity to induction of neoplastic transformation by TPA in JB-6 mouse epidermal cell lines were identified in collaboration with the Cell Biology Section, LVC. These genes were cloned by sib selection from a size-selected genomic library of clonal cells sensitive to promotion of neoplastic transformation. (1) By restriction mapping, heteroduplex analysis, direct hybridization and sequence comparisions, the *pro* genes were shown to be different genes and to have no homology known to oncogenes or other known genes. (2) The structural features of the *pro*-1 gene are highly unusual in many respects. The gene is relatively small (approximately 1 kb) and resides in a highly repeated (and possibly unstable) genomic segment containing almost all known mouse middle repeated sequences. The *pro*-1 sequence itself appears as a fusion sequence assembled from two different types of middle repetitive elements, the BAM5 and the Alu-type B1 repeat, joined by an apparently unique sequence of G4bp. The *pro*-1 sequence has all the landmark consensus sequences involved in accurate and abundant transcription employed by eukaryotic

RNA polymerase II genes. The promoter elements ("TATA", "CAAT" boxes, a typical enhancer cor sequence, and a strong ribosome binding site) are in an ordered spatial arrangement typical for pol II promoters and a poly-adenylation hexanucleotide (ATTAAA), along with a typical cleavage site, are present downstream to translation terminator coders. The open reading frame is contiguous with no introns and predicts a product of 65 amino acids (MW 7,100 daltons) with a highly unusual composition. The open reading frame (ORF) of pro-1 is now subcloned into the bacterial expression vector pJB6 as a fusion protein to lameda CII NH_2-terminal polypeptide. Experiments to express the pro-1 protein are now underway. (3) The genomic segment representing the pro-2 genes is 3.8 kb in size and is mostly a unique sequence except for a small middle repeated element present in the middle of the active gene. This repeated element is unrelated to known mouse repeats and apparently represents an unknown class of mouse repeated sequences. The frequency of repetition and marks of mobility are under investigation. Sequencing of the whole 3.8 kb active genomic fragment representing the pro-2 gene is almost complete. Computer aided analysis of the sequence for promoter elements, ORFs and mRNA processing signals will be performed. (4) Southern analyses of human, monkey and mouse DNAs have shown that both pro genes are present and, therefore, conserved during human evolution. Screening a human sperm library yielded one phage with homology to the entire pro-1 sequence, whereas the nasopharyngeal carcinoma (CNE_2 cell lines) genomic library yielded 25-30 phages, reflecting a possible amplification of the pro-1 gene in this tumor line. The phages are now under investigation.

In addition, it was found that promoter-sensitive (P^+) JB-6 cells transformed by TPA contain another active oncogene which is able to transform P^+ cells in the absence of TPA and is distinguishable from pro by restriction enzyme sensitivity.

Significance to Biomedical Research and the Program of the Institute:

Elucidation of the molecular lesions leading to the activation of oncogenes will provide in-depth understanding of cancer pathogenesis. Present studies are particularly relevant to the understanding of the mechanisms of chemical carcinogens and promoters in animal models and eventually in human carcinogenesis.

Proposed Course:

(A) Completion of current studies on chemically induced DNA changes in BALB/3T3 transformed cells by molecular cloning of the transforming gene(s) and analyzing their structure.

(B) Completion of ongoing studies on human homology of pro-1 and pro-2 genes selected from human CNE_2 nasopharyngeal carcinoma cells: both homologs will be characterized by restriction mapping, genomic organization, and biological activity as compared to mouse pro genes.

(C) Identification and cloning of transforming genes from the established human cancer cell line, e.g., human prostate carcinoma and determination of their activity in the corresponding normal epithelial cells in culture (see Project #Z01CP05276-04 LEP).

Publications:

Colburn, N. H., Lerman, M. I., Hegamyer, G. A. and Gindhart, T. D.: A transforming activity not detectable by DNA transfection to NIH/3T3 cells is detected by JB-6 mouse epidermal cells. Mol. Cell Biol. 5: 890-893, 1985.

Colburn, N. H., Lerman, M. I., Hegamyer, G. A., Wendel, E. and Gindhart, T. D.: Genetic determinants of tumor promotion: Studies with promoter resistant variants of JB-6 cells. In Bishop, M., Graves, M. and Rowley, Y. (Eds.): Genes and Cancer. Los Angeles, Alan R. Liss, Inc., 1984, pp. 137-155.

Colburn, N. H., Lerman, M. I., Srinivas, L., Nakamura, Y. and Gindhart, T. D.: Membrane and genetic events in tumor promotion: Studies with promoter resistant variants of JB-6 cells. In Fujiki, H. and Sugimura, T. (Eds.): Cellular Interactions by Environmental Tumor Promoters. Tokyo, Scientific Societies Press, 1984, pp. 155-166.

Lerman, M. I. and Colburn, N. H.: Pro genes: a novel class of genes that specify sensitivity to induction of neoplastic transformation by tumor promoters. In Cooper, G. M. (Ed.): Viral and Cellular Oncogenes. Boston, Martinus Nighoff Publishing. (In Press)

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05273-04 LEP

PERIOD COVERED

October 1, 1984 through September 30, 1985

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Molecular Mechanisms in Multistage Carcinogenesis

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	T. D. Gindhart	Expert	LEP	NCI
Others:	B. M. Smith	Guest Researcher	LVC	NCI
	N. H. Colburn	Chief, Cell Biology Section	LVC	NCI

COOPERATING UNITS (if any)

University of Texas Medical Center at Galveston, Galveston, Texas (W. R. Fleischmann); Shizuoka College of Pharmaceutical Sciences, Shizuoka, Japan (Y. Nakamura)

LAB/BRANCH

Laboratory of Experimental Pathology

SECTION

Office of the Chief

INSTITUTE AND LOCATION

NCI, NIH, Frederick, Maryland 21701

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.4	1.0	0.4

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The objective of these studies is to identify molecular mechanisms underlying specific stage transitions in multistage carcinogenesis. How transformation of preneoplastic epithelial cells depends on phosphorylation of specific proteins by the phospholipid- and calcium-dependent protein kinase (PK-C) has been the first primary question. Over 16 substrates for PK-C have been found in JB-6 cell lines. Most of them reversibly associate with the particulate fraction depending on the availability of divalent cations. All are more readily phosphorylated under cation conditions favoring phosphorylation of the basic histone, H1, (5.0 mM Ca++, 7.5 mM Mg++), but most specifically in a phospholipid dependent manner under conditions favoring the more neutral histones, H2-H4 (5.0 mM Ca++, 75 mM Mg++). One heat shock protein, pp80, is stimulated by TPA in untransformed but not in transformed derivatives of JB-6 cells. Hyperthermia induces a full heat shock response and blocks TPA promotion. The relationship between this defect in heat shock protein regulation and the synergistic antitumor effects of interferon and hyperthermia is being pursued. The generation of reactive oxygen, especially the superoxide anion, has been found to be a required event in TPA-induced promotion in JB-6 cells. Secondary free radicals implicated in this activity are OH-hydroxyl radicals and lipid peroxides but not hydrogen peroxide.

PHS 6040 (Rev 1/84)

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

T. D. Gindhart	Expert	LEP	NCI
B. M. Smith	Guest Researcher	LVC	NCI
N. H. Colburn	Chief, Cell Biol. Sect.	LVC	NCI

Objectives:

The objective of these studies is to identify molecular mechanisms underlying specific stage transitions in multistage epithelial carcinogenesis. Malignant transformation of mammalian cells by chemical carcinogens proceeds by progression through stages which can be phenotypically recognized in vivo and in parallel cell culture systems. Biochemical differences between populations of cells at successive stages can initially be utilized as stage-specific molecular markers and then exploited to analyze the underlying mechanisms responsible for progression of cells from one stage to the next. The biological model currently utilized is the mouse epidermal cell line, JB-6, with its unselected clones sensitive and resistant to transformation by promoting agents (see Project # Z01CP05383-02 LVC). Recent studies have identified genes that can determine the late-stage preneoplastic phenotype in these cell lines. Other epithelial cell systems, being characterized in other LEP projects, will be used for further studies to estimate the activity of these genes in other carcinogenesis models, e.g., in respiratory epithelia. Emphasis will be placed upon comparison of the mechanisms of tumor-promoting agents with those of carcinogens administered singly and in combinations.

This project includes two specific parts:

Part A: Determination of the roles of free radicals, especially those of reactive oxygen, in the transformation response to tumor promoters in mouse epidermal cell lines; and

Part B: Evaluation of changes in protein kinase activities as epithelial cells pass through defined stages of preneoplastic progression.

Methods Employed:

Part A: Clonal sublines of the JB-6 cell line were used to study the roles of free radicals in preneoplastic progression. The effects of eliminators of free radicals on the promotion of transformation of JB-6 cell lines by TPA have been determined. The effect of TPA on endogenous levels of superoxide dismutase was measured. An oxidant, $NaIO_4$, and an industrially common free radical generator, benzoyl peroxide, were tested for their ability to promote transformation of JB-6 cell lines.

Part B: The same battery of JB-6 clonal variants and their transformed counterparts were metabolically labeled with ^{32}P-orthophosphate and the phosphoprotein patterns of whole cell lysates and subcellular fractions were analyzed by one- and two-dimensional gel electrophoresis. Possible differences between promotion-sensitive and -resistant JB-6 cell lines in the calcium- and phospholipid-dependent protein kinase-C (PK-C) were sought by varying the conditions of the PK-C assay. Possible differences between these cell lines in substrates for this enzyme were sought in parallel by gel electrophoresis of in vitro reaction products.

Tumor-promoting phorbol esters are known to activate PK-C. The opportunity to identify TPA-induced phosphoprotein changes relevant to transformation was maximized by analyzing induced changes in the JB-6 cell lines which differ by only one or a few genes in their capacity to be transformed by TPA.

Major Findings:

Part A: As previously reported, free radical eliminators which enyzmatically catabolize the superoxide anion inhibit TPA promotion most effectively. Superoxide dismutase (SOD) and the lipophilic copper coordination compound, copper (II), (3,5-disiopropylsalicylcic acid)$_2$, with SOD-mimetic activity, inhibited TPA promotion of JB-6 cell lines up to 95% when SOD was added prior to or within four hours of exposure to TPA. SOD had no effect on the expression of the transformed phenotype, once acquired. Eliminators of hydrogen peroxide, catalase and glutathione peroxidase, enhanced expression of the transformed phenotype by 200-500%. Two antioxidants, n-propyl gallate and butylated hydroxyanisole, and two inhibitors of the lipoxygenase pathway of the arachidonic cascade, nordihydroguaiaretic acid and quercetin, inhibited TPA promotion, while an inhibitor of the cycloxygenase pathway, indomethacin, did not. These in vitro responses match those reported from studies in vivo.

TPA treatment was found to reduce the levels of endogenous SOD activity by 50% in promotion-competent clones of JB-6 cells but only by 17% in promotion-incompetent clones. This difference in suppression of SOD with TPA treatment appears to mark the promotion-competent phenotype (P^+) among JB-6 cell lines and shows greater suppression of endogenous SOD activity by TPA than does the P^- phenotype.

Transformation of JB-6 cell lines was effectively promoted both by $NaIO_4$ and benzoyl peroxide. These experimental findings were extended and analyzed to develop a general interpretative mechanism. Pathways were described whereby disruption of free-radical metabolism can account for preneoplastic progression. Testable hypotheses, thus generated, will be investigated to define the role of free radicals in tumor promotion.

Part B: A heat shock protein (pp80), deficient in tumor cell lines but increasing in response to TPA in untransformed JB-6 cell lines, was previously described.

Transformed JB-6 cell lines lack pp80 and fail to produce it in response to TPA. This finding was confirmed in studies with cell-free preparations used to study the endogenous substrates for PK-C.

PK-C enzyme activity and substrates for PK-C are similar in JB-6 cell lines of different phenotypes and derived tumor cell lines. The substrates can be categorized as histone H_1-like or histone H_2-like according to cation conditions favoring their phosphorylation. Most of them reversibly associate with the particulate fraction of cells depending on the availability of divalent cations. The TPA-sensitive heat shock protein, pp80, is not a PK-C substrate.

Recent studies by W. R. Fleischmann (University of Texas) have shown dramatic enhancement of the antiproliferative effects of gamma-interferon on B-16 melanoma cells by as little as 2°C of temperature elevation. Ongoing collaborative studies are extending analysis of this synergistic effect.

Significance to Biomedical Research and the Program of the Institute:

Identification of specific molecular mechanisms by which epithelial cells progress in multistage carcinogenesis should contribute to a more complete understanding of the pathogenesis of epithelial cancers.

Identification of PK substrates required for promotion should reveal the physiologic system which is the cellular target of tumor-promoting agents in post-initiated cells.

The heat shock protein defect found in the tumor cells suggests dysregulation of this stress resistance mechanism in malignant transformation. Heat enhancement of interferon's antiproliferative effects is a promising model to study the role of metabolic stress in relation to cell proliferation.

Identification of superoxide anion generation as a critical requirement in preneoplastic progression is relevant to many conditions and models for carcinogenesis in human and experimental systems.

Proposed Course:

(a) Identify substrates for PK-C indispensible for promotion of transformation in JB-6 cell lines. Attention will focus on substrates which can be related to *pro* gene activity, superoxide anion generation and non-phorbol ester tumor promoters.

(b) Identify the promotion-relevant targets for free radicals.

(c) Extend analysis of the synergistic antiproliferative effect of hyperthermia and interferons in treatment of cancer.

Publications:

Colburn, N. H., Lerman, M. I., Hegamyer, G. A., Wendel, E. and Gindhart, T. D.: Genetic determinants of tumor promotion: Studies with promoter resistant variants of JB-6 cells. In Bishop, M. Graves, M. and Rowley, J. (Eds.): *Genes and Cancer*. New York, A. R. Liss Inc., 1984, Vol. 17, pp. 137-155.

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Colburn, N. H., Srinivas, L., Hegamyer, G. A., Dion, L. D., Wendel, E. J., Cohen, M. and Gindhart, T. D.: The role of specific membrane and gene-level changes in the mechanism of tumor promotion: Studies with promoter resistant variants. In Borzsonyi, M., Yamasaki, H. and Hecker, E. (Eds.): The Role of Cocarcinogens and the Promoters in Human Experimental Carcinogenesis. Lyon, France, International Agency for Research on Cancer Scientific Publications, 1985, pp. 102-215.

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Gindhart, T. D., Nakamura, Y., Stevens, L. A., Hegamyer, G. A., West, M. W., Smith, B. M. and Colburn, N. H.: Genes and signal transduction in tumor promotion: Conclusions from studies with promoter resistant variants of JB-6 cells. In Mass, M. J., Kaufman, D. G., Siegfried, J. M., Steele, V. E. and Nesnow, S. (Ed.): Carcinogenesis - A Comprehensive Survey, Cancer of the Respiratory Tract: Predisposing Factors. New York, Raven Press, 1985, Vol. 8, pp. 341-368.

Gindhart, T. D., Srinivas, L. and Colburn, N. H.: Benzoyl peroxide promotion of transformation of JB6 mouse epidermal cells: inhibition by ganglioside G_T but not retinoic acid. Short Communication. Carcinogenesis. 6: 309-311, 1985.

Gindhart, T. D., Stevens, L. and Copley, M. P.: Transformation and tumor promoter sensitive phosphoproteins in JB-6 mouse epidermal cells: One is also sensitive to heat stress. Carcinogenesis. 6: 229-235, 1985.

Nakamura, Y., Colburn, N. H. and Gindhart, T. D.: Role of reactive oxygen in tumor promotion: implication of superoxide anion in promotion of neoplastic transformation in JB-6 cells by TPA. Carcinogenesis. 6: 229-235, 1985.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05274-04 LEP

PERIOD COVERED

October 1, 1984 through September 30, 1985

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Respiratory Carcinogenesis by Chemical and Physical Factors

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	U. Saffiotti	Chief	LEP	NCI
Others:	S. F. Stinson	Biologist	LEP	NCI
	R. L. Norman	Sr. Staff Fellow	LEP	NCI
	H. M. Schuller	Head, Pathology and Ultra-structural Oncology Sect.	LETM	NCI

COOPERATING UNITS (if any)

Department of Pathology, University of Maryland, School of Medicine, Baltimore, MD (E. M. McDowell, K. P. Keenan)

LAB/BRANCH

Laboratory of Experimental Pathology

SECTION

Respiratory Carcinogenesis Section

INSTITUTE AND LOCATION

NCI, NIH, Frederick, Maryland 21701

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
4.3	1.8	2.5

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Studies on the mechanisms of respiratory tract carcinoma induction by chemical and physical factors, alone or in various combinations, are pursued in the appropriate animal models. Two recently established Syrian golden hamster colonies (inbred 15:16/EHS:CR and outbred Syrian/CR:RGH) are used for intratracheal instillation of carcinogens adsorbed on particulate carriers alone or in combinations, systemic versus topical treatments, and combined treatments with carcinogens and cofactors. F-344 rats are also used for studies on mechanisms of silica-induced carcinogenesis and fibrogenesis. Respiratory epithelial tissue responses are characterized by histological, ultrastructural, histochemical and biochemical methods, and by their study in organ and cell culture conditions.

Induction of respiratory tract carcinomas by diethylnitrosamine in hamsters was found to be much more effective when the carcinogen was administered starting at one day of age than at 4 or 8 weeks, but the induction of tracheal papillomas is not affected by this age factor.

Combined exposure protocols have been used to study the effects of N-methylnitrosourea, benzo[a]pyrene (BP), 3-methylindole and dimethylsulfoxide. Studies on the role of physical factors are continuing, with protocols involving microtrauma, saline and particulate materials. Metabolism of BP was comparatively studied in hamster trachea, bronchi and lung after single or repeated instillation of BP with ferric oxide particles. The target sites for carcinogenesis (trachea and bronchi) showed the highest level of metabolism and of DNA binding.

Silica-induced pulmonary epithelial proliferative lesions are studied for their pathogenetic relationship to granulomatous cell reaction, to the role fo reactive oxygen and cellular mediators of inflammation.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

U. Saffiotti	Chief	LEP	NCI
S. F. Stinson	Biologist	LEP	NCI
R. L. Norman	Sr. Staff Fellow	LEP	NCI
H. M. Schuller	Head, Pathology and Ultra-structural Oncology Section	LETM	NCI

Objectives:

The main objective of this project is the elucidation of the mechanisms by which respiratory tract cancers, which include major forms of human cancer, are induced by chemical and physical factors, alone or in various combinations.

Studies on the pathogenesis of respiratory cancers have required the development and selection of appropriate animal models in vivo. Pathogenetic studies of these models received new emphasis, in this and other laboratories, with the development of organ culture and cell culture methods for respiratory epithelia and with their use in investigating the interaction of carcinogens with target cells (see Project #Z01CP05277-04 LEP). At present, two strains of hamsters and one strain of rats are being used in experimental studies based on selective responses of their respiratory tract epithelia.

The main biological model selected for the induction of bronchogenic carcinoma is the hamster respiratory carcinogenesis model, originally developed by intra-tracheal administration of saline suspensions of carcinogens carried by fine inorganic particles (Saffiotti, U., et al., Cancer Res. 28: 104-124, 1968). This model was subsequently extensively studied. The cellular responses and the types of tumors induced by carcinogens in the hamster model are closely similar to their human counterparts. Differentiation markers were character-ized for basal cells, intermediate cells, mucous granule cells and keratin-containing cells. The role of different carcinogens acting synergistically was identified in this system, especially for polycyclic hydrocarbons in combina-tion with N-nitroso compounds. The special role of particulate materials was identified in a series of studies with particulates having different physical characteristics as carriers of adsorbed carcinogens. These pathogenetic mech-anisms are being further investigated. An adequate induction model remains to be defined for the pathogenesis of small cell undifferentiated carcinomas.

General objectives of this project are: (a) to characterize animal models for the induction of respiratory carcinogenesis, especially in relation to their human counterpart; (b) to investigate relevant host factors such as species, age and segmental responses in the respiratory tract (nasal mucosa, larynx, trachea, bronchi and peripheral airways); (c) to study the role of combined exposures to carcinogens and cofactors; (d) to study the mechanisms involved in respiratory carcinogenesis by carcinogens adsorbed on particulate materials and the role of particulates having toxic, fibrogenic and carcinogenic effects;

(e) to analyze the metabolic pathways and DNA-binding of carcinogens in different segments of the respiratory tract and the role of cofactors; (f) to correlate in vivo effects with corresponding in vitro studies or cultured target tissues and cells.

Specific objectives are outlined in the section on "Major Findings."

Methods Employed:

Controlled breeding at the FCRF Animal Production Area, under specific pathogen-free conditions, of two colonies of Syrian golden hamsters, inbred 15:16/EHS:CR and outbred Syrian/CCR:RGH. Establishment of lifetime and serially sacrificed colony control and treatment groups with general histopathological study and special investigation of respiratory tract cell differentiation and carcinogenesis. Rats of the F-344 strain are also studied.

Intratracheal instillations of solutions and of particulate suspensions with characterization of particle distribution and retention. Study of respiratory tissue reactions by histologic, histochemical, autoradiographic and immunochemical methods and by scanning and transmission electron microscopy. Epithelial tissue isolation, fractionation and use for biochemical analysis of carcinogen localization, metabolism, and binding.

Carcinogens currently studied include polycyclic aromatic hydrocarbons and N-nitroso compounds; particulate materials currently include ferric oxide, silica (quartz, tridymite) and others to be selected.

3-methylindole (3MI) a cigarette smoke component, is being evaluated in the hamster; the time course, affected cell types and extent of damage following single or multiple injections are studied by light and electron microscopy. The effects of concurrent exposures to 3MI and polycyclic aromatic hydrocarbons, such as would occur during smoking, are being investigated in the hamster respiratory carcinogenesis model.

Major Findings:

(1) Differential age susceptibility to respiratory carcinogens: Three major long-term studies were continued in collaboration with Dr. H. M. Schuller to examine differences in type and incidence of respiratory neoplasms induced by 12 weekly subcutaneous injections of diethylnitrosamine (DEN) in hamsters which were one day, 4 weeks and 8 weeks old at initiation. Animals have been killed at 4-week intervals, and a large group is being held for lifetime observations. Proliferative changes are characterized by light and electron microscopy.

Studies in hamsters treated starting at 4 and 8 weeks have been completed and their results are being evaluated. Studies in one-day-old hamsters are nearing termination. Preliminary findings indicate that one-day-old hamsters are much more sensitive to carcinogenesis with DEN than the older animals, with respiratory tract carcinomas appearing at earlier times and in greater numbers. Incidence of tracheal papillomas, however, was similar in all age groups. Neoplasms observed (in order of decreasing incidence) were tracheal papillomas, adenocarcinomas of the ethmoid region, and adenocarcinomas of the bronchiolar-alveolar

region. A higher proportion of malignant neoplasms, especially in the nasal cavities, was found in the hamsters given DEN at the earlier age. Pathogenetic study of malignant neoplasms of the ethmoid regions of the nasal cavities suggests that they arise in the submucosal glands rather than from the olfactory epithelium as reported by previous investigators. Combined immuno-histochemical and electron microscopic investigations indicate that the lung adenomas and adenocarcinomas arise from Clara cells in the terminal bronchioles. As these lesions become more advanced they loose some of the immunologic and cytologic markers of the Clara cells, so determination of the cell of origin in larger lesions is difficult. Morphologic characterization of the cellular types is in progress.

(2) Effects of regionally selective toxic agents on the respiratory tract and lungs of hamsters: 3-methylindole (3MI) induces selective damage to non-ciliated bronchiolar cells (Clara cells) and to alveolar type I cells in some species. It was tested in pilot studies for toxicity in hamsters and currently studied for long-term effects. Hamsters killed one week after 12 i.p. doses of 25 or 50 mg/kg 3MI or DMSO (vehicle control) revealed mild focal hyperplasia of Clara cells in the terminal bronchioles only in the 50 mg/kg group. Twenty-five mg/kg 3MI or DMSO alone did not produce any significant morphologic findings in the lungs. Lifetime studies following 12 doses of 25 mg/kg 3MI in males or 15 mg/kg in females are underway. There are few intermediate deaths indicating these doses are well tolerated.

(3) Respiratory carcinogenesis with benzo[a]pyrene in inbred hamsters: Production of inbred hamsters reached the level needed for long-term carcinogenesis studies. Large groups of male and female inbred hamsters were treated intratracheally with BP-ferric oxide. The studies are at a point where respiratory tumors are beginning to appear in experimental animals treated with the carcinogen. These animals will be used for long-term tumor incidence studies. Also, at necrospy, tumor tissue is collected for correlated studies on tumor cell differentiation and culture properties. In vitro and combined in vivo/in vitro techniques are used for the study of BP metabolism in different segments of the respiratory tract.

(4) Combined effects of topical and systemic factors on respiratory carcinogenesis:

a) N-methyl-N-nitrosourea (MNU): A series of pilot studies was started to test whether various topically applied insults to the respiratory tract can enhance the respiratory carcinogenic effect of MNU given systemically. Initial lifetime pilot studies, now nearing completion, include treatments with MNU given intratracheally, combined with Fe_2O_3 given intratracheally or with 3MI given systemically. Preliminary findings from intermediate deaths do not indicate any synergism in respiratory carcinogenesis with any of the factors investigated. An additional large group of hamsters was given 10 intratracheal instillations of MNU and is being held for lifetime observations. Respiratory tumors induced in this experiment will be used for incidence studies as well as for correlated in vivo/in vitro investigations.

b) 3-Methylindole (3MI): Lifetime studies are underway on the effect of multiple concurrent treatments with BP given intratracheally and 3MI given intraperitoneally. Groups of male and female hamsters were given 12 weekly intratracheal instillations of BP-ferric oxide. Half of these were also injected i.p. with 3MI dissolved in DMSO, and the other half were injected i.p. with DMSO alone. Other groups received the BP-ferric oxide alone or the 3MI or DMSO alone. Deaths during the treatment period were usually due to pneumonia, while deaths after that time were usually associated with respiratory neoplasms. Preliminary tumor incidence studies indicate that groups which received BP-ferric oxide and 3MI or DMSO have a significantly higher incidence and earlier onset of laryngeal, tracheal and bronchogenic carcinomas, and pulmonary sarcomas as compared to groups given BP-ferric oxide alone. No difference is apparent between animals given the BP-ferric oxide together with 3MI in DMSO or with just DMSO, suggesting that DMSO may function as an important modifer of respiratory carcinogenesis in this model.

c) Combined studies on physical factors (microtrauma, saline and/or particulate instillation) with BP and/or MNU: Studies of cell injury and response have been continued with a variety of protocols, ranging from the simple microtrauma of intratracheal or intralaryngeal cannulation to the instillation of saline or of saline with Fe_2O_3 particles, with or without the addition of carcinogens. The cellular response is evaluated by histochemical and electron microscopic methods and by determining the mitotic index of various cell types. Long-term experiments reproducing these combined treatment protocols are nearing completion. This project is conducted in collaboration with the Department of Pathology, University of Maryland, under intramural support contract #N01-CP-25605 (see below for contract report).

(5) [^{3}H] Benzo[a]pyrene metabolism in vitro by tracheal and bronchial explants: Trachea and bronchus explants from inbred hamsters were incubated 20 hrs at 37° in serum-free L15 media containing 5uM BP incorporated into a phosphatidyl choline liposome. After a 2 hr incubation with beta-glucuronidase and arylsulfatase to hydrolyze conjugated metabolities, the metabolite profiles were determined by HPLC. The trachea and bronchus produced different metabolite profiles: the major trachea metabolites were 3-OH-BP, followed by the quinones, followed by the 7,8-diol and by the triol-9,10-diol; the major bronchus metabolites were quinones followed by much lower amounts of the triol-9,10-diol. Furthermore, more than 80% of the total trachea metabolites were present initially as conjugated species while only 45% of the bronchus metabolites were conjugated and that was essentially due to the quinones (87% of the increase).

On a per organ basis, the trachea produces more total metabolites than the bronchus (770 vs. 490 pmoles/20 hrs/tissue) and this difference is even more pronounced when non-quinone metabolites are compared (610 vs. 175 pmoles/20 hrs/tissue). Expressing the results per mg wet weight of tissue further accentuates this difference since the bronchus samples weighed about 50% more than the tracheas.

Hamsters were treated once a week for 12 weeks with intratracheal instillations of BP adsorbed onto ferric oxide particles. At various times after the last dose

hamsters were sacrificed and trachea and bronchus explants were incubated as described above. Tracheas removed 24 hours after the last dose exhibit a 50% increase in total BP metabolism due to increased formation of the triol-9,10 diol, 7,8-diol and 3-OH-BP. Similar increases are observed in the bronchus of these animals. However, the increases in triol-9,10-diol, 7,8-diol and 3-OH-BP observed with the bronchus are offset by a decrease in quinone production resulting in no apparent increase in total metabolism. These changes are somewhat diminished in incubations of trachea and bronchus removed from hamsters 72 hrs after the last dose, and by 6 days after the last dose the metabolite profile of both tissues resembles that of untreated hamsters. Thus, a repeated treatment protocol which yields respiratory tract tumors was found to cause a significant, albeit transient, increase in BP metabolism by the respiratory target tissues.

(6) [^{3}H] Benzo[a]pyrene binding in vivo to tissue macromolecules from the trachea, bronchi and lung: The binding of [^{3}H] BP to respiratory tract DNA of hamsters receiving 1 or 12 weekly intratracheal instillations of BP adsorbed onto ferric oxide particles was compared. (Note: Only the last dose of the 12 weekly doses was labelled with [^{3}H]). The hamsters were sacrificed at various times after the last dose and the trachea, bronchus and aliquots of the lung were extracted with acetone followed by at least 2 rounds of digestion with proteinase K and RNAase, 2 precipitations with ethanol and at least 1 treatment with collagenase. The DNA isolation was considered complete when the ratio of the sample absorbance at 260 nm to that at 280 nm equaled or exceeded 1.80. DNA from all 3 tissues was found to bind BP. When binding is expressed on a per organ basis, the lung binds more than bronchus which binds more than the trachea. This is to be expected since the lung weights are 10 times the bronchus and 35 times the trachea. However, when expressed as pmoles of BP bound per mg of DNA, the results are reversed for both treatments: the trachea binds more than the bronchus which in turn binds more than the lung. Binding was always higher to tissues from hamsters receiving 12 weekly doses than a single dose: for bronchus and lung, about 5 times higher, and for trachea at the peak, about 14 times higher.

The time course of binding was approximately the same in the bronchus and lung for both treatments: binding reached a peak at 24 to 48 hrs after the last dose and no significant decline was noted at 96 hrs or 168 hrs. A similar time course was observed with tracheas of hamsters receiving a single dose of BP ferric oxide. However, tracheas of animals receiving 12 weekly doses exhibited a binding maximum at 24 hours which declined sharply between 96 and 168 hours to near background levels. This decline could be due to the sloughing off of injured cells and the resulting cell proliferation in response to repeated trauma during cannulation. Thus, a treatment protocol which yields respiratory tract tumors was found to induce higher levels of binding of the carcinogen and the target regions at risk (the trachea and bronchi were found to have higher levels of binding than the peripheral lung tissue).

(7) Respiratory carcinogenesis with crystalline silica in rats: The recent reports of the induction of pulmonary carcinomas in rats treated with crystalline silica particles (quartz) by inhalation or by intratracheal instillation have

prompted an investigation of the early reaction of the pulmonary epithelia to silica. The alveolar and bronchiolar epithelia are found to become hyperplastic within days after intratracheal instillation of quartz dust preparations (Min-U-Sil, with or without hydrofluoric acid wash). This hyperplastic epithelial reaction was seen to persist for several months in histologic material from previous experiments and was also found to be present in lungs of rats that developed carcinomas in long-term studies in other laboratories. This epithelial reaction was found to occur in the granulomatous areas of the lung, typical of the fibrogenic nodules induced by silica and characterized by a complex cellular reaction (macrophages, fibroblasts, lymphocytes, plasma cells, mast cells, eosinophils). The role of the cellular mediators of granulomatous inflammation in the induction of epithelial proliferation and/or neoplasia is under study, both in vivo and in vitro. Mediators under study include reactive oxygen (see also Project Z01CP05173-04 LEP) and several cytokines (e.g., interleukin-1). Rats were given a single intratracheal instillation of quartz dust and were killed after various intervals or are being held for long-term observation. The cellular reactions are currently being studied.

In vitro techniques are being developed to study the role of macrophages in silica-induced carcinogenesis. Marcophages have been collected by pulmonary lavage and were maintained for up to one week in culture. Viability of the macrophages under the collection and culture conditions was verified by their ability to phagocytose quartz particles.

(8) Data reviews: Reviews were completed on the effects of particulates and other cofactors on experimental respiratory carcinogenesis, on factors involved in laryngeal carcinogenesis, on the pathology and carcinogenesis of nasal cavity, nasopharynx and upper respiratory tract tumors, and of silica-induced pulmonary lesions.

Significance to Biomedical Research and the Program of the Institute:

This project is addressed to the elucidation of the pathogenetic mechanisms of one of the major forms of human cancer; it is expected to contribute new knowledge on the conditions of concurrent or synergistic effects of different agents in respiratory cancer induction, a topic highly relevant to the understanding of human susceptibility to the multiple exposures that concur in lung cancer causation. This project is also expected to contribute basic knowledge to the poorly explored field of the mechanisms of epithelial carcinogenesis by providing an experimental pathology basis correlated with model studies on growth and neoplastic transformation of epithelial cells and on the molecular changes controlling their neoplastic transformation. New research emphasis on silica-induced carcinogenesis mechanisms is of special relevance to the investigation of the effects of recently characterized mediators of inflammation on target epithelia.

Proposed Course:

Continuation of long-term studies on respiratory carcinogenesis and its modifiers. Continuation of serial sacrifice studies for the identification of the cellular pathogenesis of preneoplastic, neoplastic and associated lesions. Extension of

investigations on the relationships between granulomatous reactions and epithelial proliferation and carcinogenesis induced by silica. Correlation of in vivo responses with studies on the metabolic fate of carcinogens in different segments of the respiratory tract. This project is closely interrelated to other projects in the laboratory on carcinogen metabolism and synergism, on epithelial cell culture and transformation, and on the identification of transforming genes in epithelial cells and in the experimentally induced tumors of the respiratory epithelia treated with different carcinogens.

Publications:

Saffiotti, U.: Connecting molecular and cellular models with the corresponding events in tissues and organs: in vitro/in vivo comparisons in respiratory tract carcinogenesis. In Proceedings of 15th Conference on Environmental Toxicology. (In Press)

Saffiotti, U.: Introduction. In Mass, M. J., Kaufman, D. G., Siegfried, J. M., Steele, V. E., and Nesnow, S. (Eds.): Carcinogenesis - A Comprehensive Survey. Cancer of the Respiratory Tract: Predisposing Factors, Vol. 8, Raven Press, New York, 1985, pp. xi-xiii.

Saffiotti, U.: Silica-induced pathology: relationships between fibrogenesis and carcinogenesis. In Silica, Silicosis and Cancer. Proceedings, International Congress, Padua, Italy, University of Padua, Specialized Regional Center on Environmental Carcinogenesis, 1985, pp. 165-181. (In Italian)

Saffiotti, U.: The pathology induced by silica in relation to fibrogenesis and carcinogenesis. In Goldsmith, D. F., Winn, D. M., and Shy, C. M. (Eds.): Silica, Silicosis and Cancer. Controversy in Occupational Medicine, Philadelphia, Praeger Publishers. (In Press)

Saffiotti, U., Stinson, S. F., Keenan, K. P. and McDowell, E. M.: Tumor enhancement factors and mechanisms in the hamster respiratory tract carcinogenesis model. In Mass, M. J., Kaufman, D. G., Siegfried, J. M., Steele, V. E. and Nesnow, S. (Eds.): Carcinogenesis - A Comprehensive Survey. Cancer of the Respiratory Tract: Predisposing Factors. New York, Raven Press, 1985, Vol. 8, pp. 63-92.

Schuller, H. M., Stinson, S. F., Ward, J. M., McMahon, J. B., Singh, G. and Katyal, S. L.: Loss of Clara cell antigens from neoplastic Clara cell derived lesions induced in the hamster lung by N-nitrosodiethylamine. Am. J. Pathol. (In Press)

Stinson, S. F. and Reznik, G.: Adenocarcinoma of the upper respiratory epithelium in the rat. In Jones, T. C., Mohr, U. and Hunt R. D. (Eds.): Monographs on Pathology of Laboratory Animals. New York, Springer-Verlag, 1985, pp. 67-71.

Stinson, S. F. and Reznik-Schüller, H. M.: Neoplasms of the mucosa of the ethmoid turbinates in the rat. In Jones, T. C., Mohr, U. and Hunt R. D. (Eds.): Monographs on Pathology of Laboratory Animals. New York, Springer-Verlag, 1985, pp. 45-53.

Stinson, S. F. and Saffiotti, U.: Experimental laryngeal carcinogenesis. In Ferlito, A. (Ed.): Cancer of the Larynx. Boca Raton, CRC Press, 1985, pp. 35-54.

CONTRACT IN SUPPORT OF THIS PROJECT

UNIVERSITY OF MARYLAND (N01-CP-25605)

Title: Hamster Respiratory Carcinogenesis Resource for In Vivo/In Vitro Correlation Studies

Current Annual Level: $393,240

Man Years: 6.9

Objectives: To conduct in vivo respiratory carcinogenesis studies in the Syrian golden hamster and to provide in vitro techniques for the culture of respiratory epithelium to complement the in vivo studies.

Major Contribution: In vivo studies: Long-term studies to investigate the interaction of chemical and physical factors in respiratory carcinogenesis are nearing completion. Groups of hamsters were given 12 weekly intratracheal or intralaryngeal instillations of saline, saline-ferric oxide, saline-ferric oxide-BP, or the above treatments preceded by a single intratracheal dose of N-methyl-N-nitrosourea (MNU). Other groups were given the MNU or intratracheal or intralaryngeal cannula insertion alone or in combination. Deaths are substantially higher in groups receiving MNU and saline-ferric oxide-BP with either intratracheal or intralaryngeal instillation than in the other treatment groups. These two treatment groups also show the highest incidence of respiratory tumors. Intratracheal instillation of the carcinogens is associated with a greater number of tracheal neoplasms as compared with intralaryngeal instillation. MNU treatment, with or without the other factors, is associated with esophageal and stomach carcinomas as well as sarcomas in various sites. In vitro studies: Various culture conditions were investigated for their abilities to maintain viability of tracheal explants for periods up to 4 weeks. Hanks or Koklik's Minimal Essential Media, with or without additives, were poor in maintaining a viable epithelium. CMRL-1066 without additives maintained normal, differentiated epithelium in the explants. Additives included transferrin, epidermal growth factor, insulin, hydrocortisone, bovine pituitary extract, ethanolamine, and phosphoethanolamine.

Proposed Course: This contract is now in its final year. Hamsters on long-term protocols will be terminated after 18 experimental months, tissues will be processed and results will be evaluated.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05276-04 LEP

PERIOD COVERED

October 1, 1984 through September 30, 1985

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Growth Control in Epithelial Cells and its Alteration in Carcinogenesis

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	M. E. Kaighn	Expert	LEP	NCI
CoPI:	D. G. Thomassen	Senior Staff Fellow	LEP	NCI
Others:	F. Bertolero	Visiting Associate	LEP	NCI
	U. Saffiotti	Chief	LEP	NCI
	M. I. Lerman	Visiting Scientist	LEP	NCI

COOPERATING UNITS (if any)

Northwestern University Medical School, Department of Urology and Surgery Laboratory of Experimental Pathology (J. Kozlowski); Center for Disease Control, Cell Culture and Medium Branch (J. Lechner)

LAB/BRANCH

Laboratory of Experimental Pathology

SECTION

Tissue Culture Section

INSTITUTE AND LOCATION

NCI, NIH, Frederick, Maryland 21701

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
3.3	1.6	1.7

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Serum-free media LEP-1 and F12/9F, respectively, were developed for mouse keratinocytes (MK) and rat tracheal epithelial (RTE) cells. Clonal growth assays were used to evaluate the basal nutrients and supplementary hormones and growth factors required for each cell type. Serum induced squamous terminal differentiation in both MK and RTE cells. Serum factors were shown to have either stimulatory (albumin) or inhibitory activity for MK cells (fetuin, crude platelet extract TGF beta). When albumin, 100 ug/ml, was added to LEP-1, MK cells continued to multiply and are now at the 33rd passage. Chromosomal studies showed an altered karyotype by the 2nd or 3rd passage and most cells were triploid to tetraploid by passage 17. This MK system in serum-free medium is currently used in studies of chemically induced transformation and gene activation.

RTE cells in F12/9F gradually stopped multiplying and underwent squamous differentiation when switched to serum-containing medium. This property of serum is used to select for preneoplastic enhanced growth variants (EG). EG variants arose spontaneously in serum-free RTE cultures. The frequency of variants increased with cell number and time. However, they appeared at a constant rate of less than one variant per one hundred thousand cells per generation. This serum-free system is being used in studies of RTE cell growth control, transformation, and differentiation.

The culture life span of a transformed but non-tumorigenic human prostatic epithelial line (NP-2s/T2) has been extended by transfection of the EJ-ras oncogene. Although the line produces a transforming growth factor, it is not tumorigenic in nude mice. Attempts to transfect normal prostatic epithelial cells (NP-2s) with known oncogenes and with DNA from prostatic carcinoma cells (PC-3) are in progress. The presence of known or new oncogenes in PC-3 is being investigated by calcium phosphate-mediated DNA transfection into NIH/3T3 cells.

PHS 6040 (Rev. 1/84)

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

M. E. Kaighn	Expert	LEP	NCI
D. Thomassen	Senior Staff Fellow	LEP	NCI
F. Bertolero	Visiting Associate	LEP	NCI
U. Saffiotti	Chief	LEP	NCI
M. I. Lerman	Visiting Scientist	LEP	NCI

(Note: This project includes continuation of research previously conducted under Projects #Z01CP05278-02 LEP and Z01CP05277-03, now terminated).

Objectives:

The overall goal of this project is to identify mechanisms controlling the growth and differentiation of normal epithelial cells and to define the sequence of changes in these mechanisms produced by carcinogenic agents. Rodent and human epithelial cell systems are used in these studies, including mouse epidermal keratinocytes, rat tracheal epithelial (RTE) cells, hamster respiratory epithelial cells and human prostatic epithelial cell lines.

Specific objectives include: (1) development of optimal culture methods for replicative growth and transformation of epithelial cells under experimentally defined conditions, particularly with serum-free media; (2) identification of exogenous factors that regulate growth and differentiation of normal epithelial cells; (3) identification of factors produced by cells that regulate their own growth (autocrine factors); (4) development of selective media for transformed cells; (5) development of optimal assays for the neoplastic transformation of epithelial cells in culture and determination of their susceptibility to different carcinogens singly or in combinations; (6) analysis of carcinogen-treated cells for changes in morphology, culture longevity, response to growth factors, alterations in enzymatic activites or structural proteins, karyotype, anchorage-independent growth, and tumorigenicity in athymic nude mice; (7) DNA-mediated transfer of transforming activity and tumorigenicity by DNA from neoplastic epithelial cells to the corresponding normal cells in culture; (8) identification of oncogenes in transformed cells; and (9) assaying for transforming growth factor production by established tumor cell lines and by carcinogen-treated cells.

Methods Employed:

(A) Mouse keratinocytes: BALB/c newborn mouse epidermal cells are isolated by cold trypsinization and separation of the epidermis from the dermis. The suspended epithelial cells are cultured in the medium, LEP-1 (Bertolero et al, Exp. Cell Res. 155: 64-80, 1984), at high density (3.5 x cells/60 mm dish) for 5-7 days, then trypsinized and frozen in liquid N_2. Clonal growth is measured by determining the average colony size after seven days of incubation. Frozen secondary cells are thawed, suspended in LEP-1 and plated in 60 mm

dishes (4 x 10^4 viable cells/4 ml). After 24 hrs the medium is removed and experimental media are added.

The cultures are incubated for six days, then fixed to determine average colony size (3 dishes, each containing 10 colonies of five or more cells are counted). The clonal growth rate is defined as population doublings/day (PD/d). Cultured cells are fixed and imbedded in situ for transmission electron microscopy (TEM) or on coverslips for scanning electron microscopy (SEM) and for immunofluorescence staining of keratin and vimentin.

For transformation experiments, the cells are exposed to graded concentrations of chemical carcinogens. Toxicity is determined by clonal survival assays. Various selection techniques are being investigated for the identification of transformed colonies, including switching from low to high Ca^{2+} concentrations in the medium or the addition of serum, to induce terminal differentiation in normal but not in transformed cells. The appearance of altered growth properties, ultrastructure (using both SEM and TEM) and karyological changes, are studied in untreated cells and as a function of time after application of the carcinogen. Growth in soft agar and tumorigenicity in nude mice are used to demonstrate acquired neoplastic properties.

(B) Rat tracheal epithelial cells: Fischer 344 tracheal epithelial cells are isolated as previously described (Thomassen et al, Cancer Res. 43: 5956-5963, 1983). For the development of a serum-free medium for normal RTE cells, Ham's F12 (modified by increasing the Ca^{2+} concentration to 0.8 mM and adding 15mM HEPES buffer) was chosen as the basal medium. Various hormone and growth factor supplements are added singly or in combination at fixed concentrations. The optimal level of each component is determined by varying its concentration while holding the levels of all other components constant. RTE cells are either cultured directly into experimental media or are switched to experimental media after 24 hrs in serum-free medium. Cultures are incubated for 10 days and the number of cells per dish is determined by routine hemocytometer count. Alternatively the cultures are fixed and stained to determine the average number of colonies of more than 30 cells.

For transformation experiments, treated or untreated cultures of RTE cells are switched to serum-containing selective medium after varying periods in serum-free medium. After four weeks more, the cultures are fixed and stained. Enhanced growth (EG) variants are identified as large colonies of small proliferating epithelial cells with an increased nuclear to cytoplasmic ratio and hyperchromasia. The frequency of transformation is defined as the percentage of RTE colonies which develop into EG variants. The rate of spontaneous transformation is calculated by the method of Capizzi and Jameson and is equal to the number of newly appearing EG variants/cell/generation.

(C) Hamster tracheal epithelial cells: Tracheas are dissected from adult Syrian golden hamsters and are cannulated with PE 160 tubing. The lumens are then flushed with phosphate buffered saline (PBS), filled with protease solution, sealed and incubated in L-15 medium at 4°C for 24 hours. Following incubation, the lumens are flushed with medium and the enzymatically dissociated cells are collected by centrifugation, resuspended in media and plated on fibronectin coated plastic dishes at 10^4 cells/cm^2.

(C) Human prostate epithelial cells: A normal human prostatic epithelial cell line (NP-2s) and a prostatic adenocarcinoma line (PC-3) are available as frozen stocks (Lechner, et al, J. Natl Cancer Inst. 60: 797-801, 1978; Kaighn, et al, Invest. Urology. 17: 16-23, 1979). Autocrine and transforming growth factors (TGFs) are isolated from serum-free medium conditioned by the cells. These factors are further purified by established biochemical methods and assayed for TGF activity in soft agar using NP-2s and NRK cells as indicators. The transforming activity of DNA isolated from PC-3 or other lines, e.g., carcinogen-treated NP-2s, are transfected into NP-2s and NIH/3T3 cells by calcium phosphate/DNA precipitation. The protoplast fusion technique (in collaboration with J. F. Lechner) is also used with cloned oncogenes. Putative oncogenes are isolated from PC-3 cells and DNA-transfected cells by established methodology (see Project #Z01CP05265-04 LEP). The metastatic activity of PC-3 cells is determined by inoculation of cells into nude mice (in collaboration with J. M. Kozlowski).

Methods are being tested and developed that will select for NP-2S transfected cells. Both resistance to the antibiotic G418, conferred by the plasmid pSV2neo, and mycophenolic acid, conferred by the xanthine-guanine phosphoribosyl transferase gene (gpt), are in use. Selection of transformed (oncogene-altered) cells by growth in serum (see sections on RTE cells and MK cells) is also being evaluated.

Major Findings:

(A) Mouse keratinocytes: Because of the difficulties encountered when serum-supplemented media were used to culture primary mouse epidermal keratinocytes (MK), a serum-free formulation (LEP-1) was developed (Bertolero et al, Exp. Cell Res. 155:64-80, 1984) consisting of Ca^{2+}-free Eagle's MEM with nonessential amino acids and seven added factors: hydrocortisone, $5x10^{-7}$ M; insulin, 5 ug/ml; phosphoethanolamine and ethanolamine, each $5x10^{-5}$ M; transferrin, 5 ug/ml; epidermal growth factor, 5 ng/ml; bovine pituitary extract, 180 ug of protein/ml. This medium was found to have a Ca^{2+} concentration of 0.03 mM by atomic absorption. In addition to undergoing a useful number of population doublings (20 or more), keratinocytes cultured in LEP-1 retain the capacity to terminally-differentiate when the Ca^{2+} concentration of the medium is increased beyond 0.1 mM.

Even though satisfactory growth of mouse keratinocytes was obtained in serum-free LEP-1, it was important to understand the role of serum factors in controlling their growth and differentiation, because normal and transformed cells respond differently to serum components and this medium is being used in transformation experiments. Recently, Lechner and coworkers have shown that the growth of normal human bronchial cells is inhibited by serum in a dose-dependent fashion. In contrast, serum stimulated while serum-free medium failed to support the growth of cell lines derived from lung carcinomas. Experience has shown, in this laboratory and others, that the overall effect of most batches of serum is due to the combined action of stimulatory and inhibitory factors.

Serum-free LEP-1, was used to investigate the control of growth and differentiation by serum factors. The addition of 3% whole fetal bovine serum (FBS) to primary and secondary cultures of confluent MK cells dramatically altered cell morphology. Cells became enlarged, flat and tightly apposed to one another with

characteristic features of squamous terminally-differentiating keratinocytes within 24 to 48 hrs of exposure to wFBS. They detached from the dish within 6 days of exposure to wFBS. Their morphology in wFBS was similar to that observed in higher levels of calcium Ca^{++} (>0.1 mM). In the clonal assay the colony forming efficiency (CFE) was reduced to 60% that of controls in 0.5% wFBS (155 ug FBS protein/ml). The surviving colonies showed squamous differentiation. When stripped FBS was titrated in the clonal assay, concentrations above 0.25% inhibited the CFE in a linear, dose-dependent manner. The resulting slope closely resembled that obtained in response to wFBS. In contrast, the response of MK cells to chelexed FBS was biphasic, since low concentrations (up to 0.5% v/v) stimulated the CFE, while higher concentrations (>0.7% v/v) were inhibitory, although to a lesser extent than equivalent concentrations of wFBS and stripped FBS.

Albumin (BSA) and fetuin (FET), the two major protein components of FBS, were tested and found to have opposite effects on MK cells. Increasing concentrations of BSA increased the CFE up to 3.5-fold at 500 ug/ml and also increased the growth rate from 0.68 to 0.86 PD/d. Cell morphology remained compatible with that of replicating basal keratinocytes and no features of squamous terminal differentiation were observed. In contrast, the addition of either FET or HDL decreased the CFE in a linear dose-dependent manner, more so for HDL than FET at equivalent concentrations.

Commercial platelet-derived growth factor preparations strongly inhibited the CFE of MK cells. Two lots from the same supplier were tested with the same results. When confluent cultures of secondary keratinocytes were exposed to 1 U/ml of commercial PDGF their morphological appearance changed from that characteristic of growing basal cells to that of squamous differentiating cultures. Work from other laboratories has implicated TGF beta, a major component of blood platelets, as an inhibitory factor present in serum and other tissues and cells. Preliminary titration experiments with secondary MK cells indicate that TGF beta (obtained from Dr. Michael Sporn, DCE, LC, NCI) is highly inhibitory (10 pg/ml gave <10% colony survival).

As a result of the serum factor studies outlined above, BSA, 100ug/ml was added to the formulation of LEP-1. In this medium, MK cells at passage 5 have continued to multiply and are currently at the 33rd passage. Cells were exposed to 5% wFBS or 1.0 mM Ca^{2+} at the 17th passage. In contrast to early passage cells, they grew under both conditions although their appearance became more irregular and less epithelial-like. Because of this increased growth potential, the karyotype of passage 17 was studied by Giemsa banding. Analysis of 100 metaphase preparations showed 6 with 40-43 chromosomes and 94 with 78-84 chromosomes. Several minutes and double minutes were also seen. Very few diploid or near-diploid metaphases were present in the cell population and those that were found were not normal in all respects. Normal chromosomes were present, but the diploid number of each chromosome was not. A more detailed analysis was carried out on two metaphase spreads. In one karyotype, chromosome #4 is monosomic, while chromosomes #6 and #19 are trisomic. These gains and losses appear to be random from inspection of photographs made of other metaphases. A normal diploid metaphase could not be found.

In the other karyotype (81 chromosomes), the chromosomes were normal in appearance but not in number for all. In this instance, chromosome #16 was triploid, while

five copies are present of chromosome #19. In the counts previously reported, all of the high ploidy cells were not perfect tetraploids, and a perfect tetraploid could not be seen. However, there seems to be little Robertsonian fusion, since only 1 of 30 metaphases scanned had a metacentric chromosome. This karyotype is representative of the metaphases in this cell line, i.e., near tetraploid, with random loss and gain of normal chromosomes. Chromosome studies on very early passages (2-4) are much more difficult because of the fragility of the cells and fewer mitotic cells. However, preliminary evidence indicates that the normal diploid karyotype is lost within 2-3 passages. Others have reported similar early chromosome changes in MK cells in serum-containing media. It appears that the rapid loss of diploidy is not a result of serum but rather is an intrinsic characteristic of the mouse. Experiments are underway to determine whether the observed karyotypic changes are accompanied by alterations in growth control and nutritional or growth factor response.

(B) Rat tracheal epithelial cells: In the previously described transformation system (Thomassen et al, Cancer Res. 43: 5956-5963, 1983) RTE cells were grown in serum-containing medium on irradiated feeder cells. To eliminate the undefined roles of serum and feeder cells in the growth and transformation of RTE cells a serum-free medium was developed. As a starting point, Ham's F12 with 0.8M Ca^{2+} and 15 mM HEPES was used. Several hormones and growth factors in use in other studies were tested as supplements to substitute for serum. Each medium formulation was evaluated by a clonal assay for colony forming efficiency (CFE). No single factor was found to be essential for the clonal proliferation of RTE cells; rather deletion of individual factors decreased either the number or size of colonies. Multiple deletions revealed interactions between factors. For example, exposure of RTE cells to pituitary extract for as little as 24 hrs was found to be important for maximal colony formation and proliferation. However, pituitary extract has some inhibitory effects as well which can be counteracted by bovine serum albumin and cholera toxin. In contrast to the lot-to-lot variability previously experienced with serum, at least 5 independent preparations of bovine pituitary extract were nearly identical in promoting maximal colony forming efficiency of RTE cells at equivalent doses (protein concentrations). Triiodothyronine (T3) given to RTE cells at the time of culture or 24 hrs thereafter increased CFE 2- to 5-fold in the presence of pituitary extract. TPA (12-O-tetradecanoyl-phorbol-13-acetate) added at the time of culture or up to 12 hrs later increased CFE 3- to 5-fold with or without pituitary extract. TPA had no effect if added 24 hrs after primary culture. Three retinoids (retinyl palmitate, retinyl acetate and retinol) were stimulatory at culture (<40% increased CFE) and inhibitory if given 24 hours later (>15% decreased CFE). Retinoic acid was inhibitory at both times with a greater effect at 24 hrs after plating. Additional factors (insulin, hydrocortisone, epidermal growth factor, transferrin) were found to support maximal cell proliferation and colony formation when added 24 hrs after culture.

The serum-free medium presently in use for transformation experiments consists of Ham's medium F12 with 0.8mM Ca^{2+} and 15mM HEPES buffer supplemented with 9 factors (epidermal growth factor, 5 ng/ml; transferrin, 5 ug/ml; insulin, 5ug/ml; hydrocortisone, 0.5uM; ethanolamine and phosphoethanolamine, 50 uM each; cholera toxin, 1nM; bovine pituitary extract, 1% (v/v); BSA, 500ug/ml). In this medium, RTE cells retain epithelial morphology and stain for keratin with anti-keratin antibody. On the ultrastructural level they have surface microvilli,

cytoplasmic filaments, desmosomes and tight junctions. RTE cells also have a population doubling time of 22 hrs, form colonies at high or low plating density with an efficiency of 2-5%, and are capable of sustained proliferation and/or maintenance of epithelial cell and colony morphology for more than 4 wks in this serum-free medium.

In previous studies on carcinogen-induced transformation of RTE cells, serum was present in the medium conditioned by 3T3 feeder cells. With the availability of a serum-free formulation for RTE cells, it was important to determine whether serum might play a role in transformation. Since it had been shown that serum inhibited the growth and stimulated differentiation of normal RTE cells, whereas EG variants were able to grow in its presence, serum was used as a selective agent. The spontaneous transformation rate in serum-free medium was determined by imposing serum selection after increasing times under serum-free conditions. Both cell number (>100X) and frequency of appearance of EG variants (>50x) increased as a function of the time of selection (3-23 days). In contrast, the calculated rate of spontaneous development of EG variants with time remained constant within a factor of two at 6×10^{-6} variants/cell/generation. This observation illustrates the significance of cell proliferation in the spontaneous development of neoplastic potential. Next it was found that treatment of serum-free cultures with the carcinogens, MNNG, benzo[a]pyrene and benzo[a]pyrene diol-epoxide, induced EG variants selectable in serum-containing medium.

Since both spontaneous and carcinogen-induced transformation of RTE cells had been demonstrated in serum-free medium, a comparison was made between the transformation of RTE cells grown in serum-free medium and on irradiated feeder cells in serum-containing medium. MNNG was considerably more cytotoxic and potent as a transforming agent to RTE cells grown in serum-free medium than when feeder cells and serum were present. A dose of 0.1ug MNNG/ml was nontoxic and nontransforming to RTE cells growing on feeder cells in serum-supplemented medium, whereas the same carcinogen dose in serum-free medium was highly toxic (<20% survival) and almost maximally transforming (2.3%). However, the maximum frequencies of transformation observed under these two culture conditions were the same (>3.5%) although the effective inducing doses of MNNG differed.

The nature of the serum-mediated selection for EG variants was examined. Preliminary experiments indicate that the antiproliferative activity of serum is reversible as long as 2 wks after switching to serum-containing medium. In addition, serum is stimulatory to normal RTE cells if given in the presence of insulin, hydrocortisone, epidermal growth factor and cholera toxin. A possible explanation for the selective growth of variants in serum supplemented medium might be that the variants have lost a requirement for a growth factor(s) absent from the serum. Alternatively, the serum could contain factors that inhibit growth of normal cells and to which the variant cells have become non-responsive. In addition to its well-known complement of mitogenic factors, serum has been shown to contain factors that inhibit growth and stimulate terminal differentiation in a variety of cell types (see section on MK cells). The factor in serum responsible for this action has been identified as TGF beta (Lechner, et al, Cancer Res. 43: 5915-5921, 1983; and T. Masui personal communication). The role of the transforming growth factor beta (TGF beta) in the serum-mediated differentiation of RTE cells is also being examined. TGF beta (obtained from Dr. Michael Sporn, LC, DCE, NCI) induced a dose-dependent

squamous differentiation of RTE cells, rducing colony formation by >90%. In contrast, the same doses of TGF beta did not inhibit colony formation by an EG variant cell line. The roles of TGF beta and the factor deficiency of serum in serum-mediated selection for EG variants of RTE cells is being investigated further.

(C) Hamster tracheal epithelial cells: Because of the advantages of using serum-free media, the efficiency of various formulations for the explant culture of hamster respiratory epithelial was explored. Serum-free Ham's F-12 with seven growth factors (insulin, hydrocortisone, ethanolamine, phosphoethanolamine, transferrin, epidermal growth factor, and bovine brain extract) was used to culture tracheal rings as organ explant cultures. The same medium was also used to culture hamster tracheal epithelial cells suspension isolated by protease digestion. Coating of culture dishes with a mixture of collagen, fibronectin and BSA enhanced attachment of the isolated tracheal cells, when plated in Ham's F-12 medium without serum or growth factors.

(D) Human prostatic epithelial cells: Because of the increased interest in prostatic cancer, a major form of cancer in man, we recently have begun a concentrated effort to investigate the mechanisms of carcinogenesis and metastasis in human prostatic epithelial cells taking advantage of the normal and neoplastic prostatic cell lines available (see Methods Employed). The difficulty in transforming normal human cells with chemical carcinogens is well known. This problem has been approached using normal prostatic epithelial cells as a model in collaboration with Dr. John F. Lechner (see project #Z01CP05130-04 LHC). A line of normal human prostatic epithelial cells (NP-2s) had previously been transformed by SV-40 (NP-2s/T2). Although this line had altered properties, including aneuploidy, anchorage independence, reduced serum requirement for clonal growth, and increased culture longevity, it has never acquired the capacity for either unlimited growth or tumorigenicity in athymic nude mice. NP-2s/T2 cells were either infected with Kirsten sarcoma virus or transfected via protoplast fusion with a derivative of plasmid pSV2*gpt*$^{+}$ carrying the EJ-*ras* oncogene. Both the Kirsten virus-infected and EJ-*ras*-transfected cells have the *ras* oncogene incorporated into their nuclear DNA. In addition, both of these *ras*-oncogene-containing cultures elaborate a transforming growth factor demonstrated by the induction of growth in soft agar of normal rat kidney (NRK) cells. However, neither line to date has become tumorigenic in nude mice and even though their lifespans were at least doubled, they eventually stopped multiplying. These findings have underscored the role of oncogenes in extending the life span of prostatic epithelial cells and may provide a means to analyze the stages leading to tumorigenicity.

In collaboration with M. I. Lerman, (see project Z01CP05265-04) an effort is being made to transfer genetic information from a metastatic prostatic adenocarcinoma line (PC-3) to normal prostatic epithelial cells (NP-2s) by transfection of tumor cell DNA. Thus far whole tumor DNA and several cloned oncogenes have been used. Extension of culture lifespan is being used as an initial index. Preliminary attempts to insert a selectable marker, the neomycin-resistance gene (pSV2neo) by transfection, have been complicated by the fact that the normal NP-2s cells are sensitive to both the calcium phosphate used for

transfection and the selective agent, G-418. As an alternative method we are evaluating the use of the gpt gene that codes for xanthine-guanine phosphoribosyl transferase and permits cells to grow in mycophenolic acid. Experiments are also in progress to determine whether high molecular weight DNA extracted from the prostatic carcinoma line, PC-3, has transforming activity in NIH/3T3 cells. In the first attempt, PC-3 DNA was transfected into NIH/3T3 cells by calcium phosphate precipitation without selection. A significant increase in the number of foci in treated over control plates was observed. Several foci were subcultured and expanded. Their DNA has been isolated and purified and is presently being examined for the presence of human alu sequences with a P^{32} alu probe. PC-3 DNA apparently does not contain altered ras oncogenes.

The metastatic capability of the prostatic adenocarcinoma line (PC-3) in nude mice has been investigated in collaboration with Dr. James M. Kozlowski (University of Chicago). The incidence of metastasis was increased by the isolation of variant sublines from secondary tumor deposits, by the use of an advantageous inoculation site and by prolonged administration of - estradiol to suppress natural killer cell activity. Intrasplenic injection resulted in the most dramatic expression of metastasis, giving large and frequent metastases to the liver and lungs as cell and to the mesenteric, omental and mediastinal lymph nodes. For example, a PC-3 variant (PC-3-M) isolated from a lung metastasis produced 13 times the incidence of pulmonary metastasis as did the parental line after i.v. injection. Direct intrasplenic injection of PC-3 gave an incidence of metastasis of 16/20 in the lung, 20/20 in the liver and 13/20 in lymph nodes. The basis of this enhancement of metastatic capability in variant lines is unknown. The fact that alternate metastatic phenotypes can be selected may be correlated with different responses to growth factors, substrates and even activated or amplified oncogenes. Experiments with a PC-3-M line metastatic to the liver are being initiated.

In serum-free medium, the growth of PC-3 is population-dependent, indicating the secretion of an autocrine growth factor. An effort will be made to isolate and purify this factor from PC-3 cells. It is not known whether this autocrine activity has TGF activity. The standard used for comparison will be TGF beta. Induction of a TGF by PC-3 DNA in normal cells would provide an important model for the genetic control of neoplastic growth by a specific gene product.

Significance to Biomedical Research and the Program of the Institute:

Since epithelial cells give rise to the vast majority of human cancers, an understanding of their mechanisms of carcinogenesis is critically important. Cancer of the skin, respiratory tract and prostate are important forms of human cancer, for which relevant animal models have been well characterized. Both rodent and human epithelial culture systems have been developed to investigate the action of etiologic factors and carcinogenic mechanisms in these cell types. The mouse keratinocyte culture system is a counterpart to the well-established in vivo model of carcinogenesis, and offers the opportunity of analyzing the action of carcinogens in epithelial cells that exhibit terminal differentiation responses of general significance. Rat tracheal epithelial cells also terminally differentiate. In this system it has been possible to use normal, preneoplastic and neoplastic cells, all derived from the same original cell population, to study

the action of chemical carcinogens and to begin to identify and isolate genes relevant to the development of neoplasia. These two rodent models should provide a means to study the cellular and molecular events leading to carcinogenesis in two major epithelial systems. The establishment of serum-free culture systems for these epithelial cell types has made it possible to study them in the absence of undefined but biologically active serum factors.

Prostatic cancer is a major form of cancer in older men. The availability of normal epithelial, as well as neoplastic human prostatic cell lines, offers an opportunity to investigate etiologic factors and mechanisms of carcinogenesis and metastasis in these human epithelial cell types using approaches similar to those used with the rodent models.

Proposed Course:

Following the development of selected culture conditions for human and rodent epithelial cell systems, attention is now primarily directed toward the investigation of sequential changes produced by oncogenes and by carcinogens. In the mouse keratinocyte system, optimal conditions for initial transformation and progression to neoplasia will be investigated using treatments with chemical carcinogens, promoters and known oncogenes, singly and in combinations. In an effort to identify stages in the neoplastic process, alterations in cellular responses to growth factors will be assessed and used as markers to develop selective media for carcinogen- or oncogene-altered cells. For example, since mouse keratinocytes require BPE for growth in LEP-1, it will be determined whether BPE-independent lines will develop after treatment with carcinogens. Since the nature of the growth-promoting activity in BPE is unknown, an effort is under way to identify the factor(s) involved.

Serum-free culture medium suitable for the growth and transformation of rat tracheal epithelial has recently been developed. Attention will now be directed toward the improvement and utilization of this system to gain further insight into the mechanism of epithelial cell carcinogenesis. As in the case of mouse keratinocytes, efforts will be made to replace bovine pituitary extract, the major undefined component of the serum-free culture system with known factors found in the pituitary. The role of modulators of transformation such as T3 and retinoids will be investigated. The roles of serum and TGF beta and the specific growth factor requirements of EG variants will be examined in an effort to understand the cellular changes which make possible the use of serum to select for EG variants. To study the role of differentiation in RTE cell transformation, an assay using cornified envelope production for RTE cell differentiation will be developed. Finally, studies will be initiated in conjunction with Project #Z01CP05381-02 LEP to examine the role(s) and relationship(s) of exogenous oncogenes and endogenous cellular homologues of oncogenes to RTE cell proliferation, differentiation, and transformation.

A new line of investigation is being developed in an effort to identify the stages required to transform human diploid prostatic epithelial cells. Normal prostatic epithelial cells will be treated with chemical carcinogens and/or selected oncogenes. Changes in culture life span, response to growth-factors and induction of anchorage independence will be monitored. Continued efforts

will be made to transform normal cells (NP-2s) by transfection of DNA extracted from prostatic carcinoma cells (PC-3). The presence and identity of known or possibly new oncogenes in PC-3 cells will be further investigated by transfection of NIH/3T3 cells. Because PC-3 cell growth is population dependent in defined medium (Kaighn, M.E., et al., Proc. Natl. Acad. Sci. USA 78: 5673-5676, 1981), it produces an autocrine factor. Since the PC-3 cell line is anchorage independent, growth in soft agarose using NRK and NP-2s cells as targets will be used to determine whether medium in which this line has been growing has tranforming growth factor activity.

The incidence of metastatic activity of PC-3 in nude mice was enhanced by isolation of variant sublines from secondary tumors. The basis of this metastatic enhancement will be investigated by correlating the response of these variant lines to growth factors and potential activation or amplification of oncogenes.

Publications:

Bertolero, F., Kaighn, M. E. and Saffiotti, U.: Mouse epidermal keratinocytes: Clonal proliferation and response to hormones and growth factors in serum-free medium. Exp. Cell Res. 155: 64-80, 1984.

Kozlowski, J. M., Fidler, I. J., Campbell, D., Xu, Z., Kaighn, M. E. and Hart, I. R.: Metastatic behavior of human tumor cell lines grown in the nude mouse. Cancer Res. 44: 3522-3529, 1984.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05381-02 LEP

PERIOD COVERED

October 1, 1984 through September 30, 1985

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Cellular/Molecular Stages of Carcinogenesis in Respiratory Epithelia

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	D. G. Thomassen	Senior Staff Fellow	LEP	NCI
Others:	U. Saffiotti	Chief	LEP	NCI
	M. E. Kaighn	Expert	LEP	NCI
	M. I. Lerman	Visiting Scientist	LEP	NCI

COOPERATING UNITS (if any)

Laboratory of Pulmonary Function and Toxicology, NIEHS, NIH, Research Triangle Park, NC (P. Nettesheim, J. C. Barrett, T. Gilmer); Molecular Mechanisms of Carcinogenesis Laboratory, Litton Bionetics, Inc., Frederick, Md. (Y. Ito)

LAB/BRANCH

Laboratory of Experimental Pathology

SECTION

Tissue Culture Section

INSTITUTE AND LOCATION

NCI, NIH, Frederick, Maryland 21701

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.6	0.8	0.8

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The objective of this project is to identify and characterize changes at the cellular and molecular levels responsible for the development of preneoplastic and neoplastic variants of rat tracheal epithelial (RTE) cells. To identify cells having critical preneoplastic changes, comparative transformation experiments are described in which independent populations of preneoplastic RTE cells are treated with N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) or with cloned oncogene-containing DNAs from Harvey murine sarcoma virus (HaMSV, ras), polyoma virus, and MC29 virus (myc). Differential responses of the various cell lines to these treatments will be used as evidence of differences in their preneoplastic potential. Using the hypothesis that differential responses of "preneoplastic" cell lines to carcinogens or oncogenic DNAs have a genetic basis, efforts will be directed towards the molecular cloning of the genes responsible for the conversion of preneoplastic to neoplastic cells. These studies require the development of assays suitable for the detection of these genes. The use of preneoplastic RTE cells for identification of RTE cell tumor genes is described. This assay for tumor genes will provide an intraspecific system in which the genes will be identified in cells that are genetically related to the cells in which the gene was originally active. Strategies are planned for cloning RTE cell tumor genes. Transfections of normal RTE cells with cloned oncogene-containing DNAs are also described.

PHS 6040 (Rev 1/84)

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

D. G. Thomassen	Senior Staff Fellow	LEP	NCI
U. Saffiotti	Chief	LEP	NCI
M. E. Kaighn	Expert	LEP	NCI
M. I. Lerman	Visiting Scientist	LEP	NCI

Objectives:

The overall goal of this project is to provide evidence on multistage carcinogenesis by identifying critical preneoplastic intermediates using the rat tracheal epithelial (RTE) cell system. Through the use of DNA transfection of cloned oncogenes, the role(s) and relationship(s) of exogenous oncogenes and the endogenous cellular homologues of oncogenes to RTE cell proliferation, differentiation, and transformation will be examined.

Specific short-term objectives are: (1) comparison of preneoplastic rat tracheal epithelial (RTE) cell lines, which differ in their ability to progress to neoplasia, for the inducibility of neoplastic potential by treatment with the carcinogen, N-methyl-N'-nitro-N-nitrosoguanidine (MNNG), or by transfection with oncogenic DNAs; (2) to develop an assay using preneoplastic RTE cells to detect genes transferring the tumorigenicity phenotype; (3) to transfer the tumorigenicity phenotype by transfection using DNA from RTE tumor cells; (4) to begin cloning a gene from an RTE tumor cell which encodes the tumorigenicity phenotype; and (5) to transfect normal RTE cells with oncogenic DNAs and to compare the phenotypes obtained with those found after transformation of RTE cells with chemical carcinogens.

Methods Employed:

Neoplastic RTE cells are identified using the athymic nude mouse tumor assay. Athymic NCr nude mice are injected subcutaneously with cell suspensions and monitored for tumor formation. Treatment of RTE cells with MNNG are done using a 4-hour treatment in HEPES buffered F12 medium without serum. DNA transfections using cloned oncogenes or high molecular weight DNAs are done using the calcium-phosphate DNA precipitation technique. Transfection of normal RTE cells are done using culture conditions described in LEP Project #Z01CP05276-03. Clones of oncogene-transfected RTE cells are isolated following cotransfection of oncogene and pSV2-neo DNA and selecting for antibiotic G418-resistant clones. Carcinogen or DNA-treated cells are assayed in nude mice for the induction of neoplastic potential. Molecular analyses of oncogene-transfected RTE cells are done using DNA and RNA isolation, RNA dot blotting, enzyme restriction of DNA, separation of DNA on agarose gels, Southern blotting, and Western blotting for some oncogene-encoded proteins. Techniques used for molecular cloning include those listed above in addition to separation of DNA fragments on sucrose gradients and construction and screening of partial genomic libraries.

Major Findings:

Transfection of preneoplastic RTE cell lines, enhanced growth (EG) variants, with molecularly cloned oncogene-containing DNAs gave variable results depending on the EG variant and oncogene(s) involved. Three nontumorigenic EG variants transfected with a genomic clone of polyoma virus (obtained from Y. Ito, Litton Bionetics, Inc.) containing genes for large T, middle T, and small T antigens all formed tumors rapidly when injected into nude mice. In contrast, transfection of the same three EG variants with a genomic clone of Harvey murine sarcoma virus containing the viral Harvey ras (v-Ha-ras) oncogene resulted in tumor formation by cells from only one of the three treated EG variant lines. This result is in contrast to previous reports using different preneoplastic fibroblastic cell lines which became neoplastic after treatment with the ras oncogene. The basis for the differential response of these EG variant lines is under investigation.

Normal RTE cells in serum-free culture (see LEP Project #Z01CP05276-03) could serve as recipients for exogenous DNA in transfection experiments. Transfection of DNA (pSV2-neo) encoding resistance to the antibiotic G418 into RTE cells resulted in antibiotic resistant colonies only in pSV2-neo treated cultures. Cotransfection of pSV2-neo and polyoma virus DNAs into normal RTE cells yielded antibiotic resistant colonies having focal areas of morphologically altered cells which were highly refractile, tightly packed and piled up. In addition, these colonies grew in both serum-free and serum-containing media unlike normal RTE cells which only proliferate in serum-free medium. When cells from these altered colonies were passaged, squamous differentiation, typical RTE cells, and focal areas of morphologically altered cells were all seen. The tumorigenicity and expression of polyoma virus antigens in these cells are being examined.

DNAs from a tumorigenic EG variant line and from a nontumorigenic EG variant line were used to transfect NIH 3T3 cells. Foci of morphologically altered cells formed in cultures treated with tumorigenic but not nontumorigenic DNA. Foci were isolated from these cultures and the DNA from these cells is being used in secondary transfections and to molecularly clone the EG variant tumor gene.

Significance to Biomedical Research and the Program of the Institute:

Epithelial cells, especially respiratory epithelial cells, represent a major target for carcinogens and for the development of neoplasia in vivo. The development of the rat tracheal epithelial cell culture system provides the opportunity to: (a) simultaneously study normal, preneoplastic, and neoplastic cells; (b) determine the effects of chemical carcinogens: (c) examine the role of oncogenes; and (d) identify and isolate genes involved in the development of neoplasia. The opportunity to study all stages of neoplastic development in one cell type at both the cellular and molecular levels should provide new insights into the mechanisms of carcinogenesis in a major epithelial tissue.

Proposed Course:

1) Having demonstrated a differential response of EG variant lines to neoplastic transformation by v-Ha-ras, this response will be further verified and characterized. The v-Ha-ras treated cells will be analyzed for the presence and expression

of the v-Ha-*ras* gene at the DNA, RNA, and protein levels. In addition the relative probabilities of these different cell lines progressing to neoplasia spontaneously or with additional carcinogen treatment will be determined.

2) The transforming potential of exogenous oncogenes in normal RTE cells will be compared to that described in LEP Project #Z01CP05276-04 for chemical carcinogens.

3) The role of the 3 polyoma virus oncogenes in the neoplastic transformation of RTE cells will be examined. Previous reports with fibroblastic cell systems have suggested that two oncogenes were sufficient for neoplastic transformation. The preliminary results reported here for epithelial cells suggest that additional changes are necessary.

4) The isolation, molecular cloning, and characterization of an RTE cell tumor gene will be continued.

Publications:

None.

ANNUAL REPORT OF

THE LABORATORY OF HUMAN CARCINOGENESIS

NATIONAL CANCER INSTITUTE

October 1, 1984 to September 30, 1985

The Laboratory of Human Carcinogenesis conducts investigations to assess (1) mechanisms of carcinogenesis in epithelial cells from humans and experimental animals, (2) experimental approaches in biological systems for the extrapolation of carcinogenesis data and mechanisms from experimental animals to the human situation, and (3) host factors that determine differences in carcinogenic susceptibility among individuals.

The scientific and managerial strategy of the Laboratory is reflected in its organization into three sections, i.e., In Vitro Carcinogenesis Section (IVCS), Carcinogen Macromolecular Interaction Section (CMIS), and Biochemical Epidemiology Section (BES). Scientifically, the emphasis is on the role of inherited or acquired host factors as important determinants in an individual's susceptibility to environmental or endogenous carcinogens and cocarcinogens. Our investigations of host factors involve interspecies studies among experimental animals and humans, cover the spectrum of biological organization ranging from molecules to the intact human organism, and are multidisciplinary, including molecular and cellular biology, pathology, epidemiology, and clinical investigations. Two sections (IVCS and CMIS) devote their major efforts to more fundamental and mechanistic studies. The scientific findings, techniques, and concepts developed by these two sections and, of course, the scientific community at large, are utilized by the BES in selected and more applied studies of carcinogenesis and cancer prevention. The laboratory-epidemiology studies in this section require the expertise found in the IVCS and CMIS and in the NCI Epidemiology Program. Resources needed by the Laboratory are unique and complex. For example, collection of viable normal as well as neoplastic epithelial tissues and cells--well characterized by morphological and biochemical methods from donors with an epidemiological profile--requires the continued cooperation among donors and their families, primary care physicians (internists, surgeons, house staff), surgical pathologists, nurses, epidemiologists, and laboratory scientists.

CULTURE OF HUMAN TISSUES AND CELLS

Remarkable progress has been made during the last few years by this and other laboratories in establishing conditions for culturing human epithelial tissues and cells. Normal tissues from most of the major human cancer sites can be successfully maintained in culture for periods of weeks to months. We have developed chemically defined media for long-term culture of human bronchus, colon, esophagus, and pancreatic duct. Primary cell cultures of human epithelial outgrowths have been obtained from many different types of human tissues. Isolated epithelial cells from human bronchus and esophagus can be transferred three or more times and can undergo more than 30 cell divisions. Human bronchial and esophageal epithelial cells can also be grown in serum-free culture medium. Morphological, biochemical, and immunological cell markers have been used to identify these cells as unequivocally of epithelial origin.

Clonal growth of normal human pleural mesothelial cells in a low serum culture medium has also been achieved so that the in vitro transformation, by asbestos, of these cells can be studied.

The availability of nontumorous epithelial tissues and cells that can be maintained in a controlled experimental setting offers an opportunity for the study of many important problems in biomedical research, including carcinogenesis. For example, the response of human bronchial epithelial cells (enhanced growth or differentiation) after either exposure to carcinogens and/or tumor promoters or DNA transfection by oncogenes is being actively investigated. Parallel investigations using epithelial tissues and cells from experimental animals allow investigators to study interspecies differences in response to carcinogens, cocarcinogens, and anticarcinogens.

CELLULAR GROWTH AND DIFFERENTIATION

Our operational definitions of normal, premalignant, and malignant cells are biological (e.g., differentiated state, growth, altered cellular affinities and architecture) and tumorigenicity when injected into the appropriate host. Methods for the culture of human epithelial tissues and cells provide an opportunity to investigate the biology and molecular mechanisms of carcinogenesis directly in human target cells and to conduct studies comparing carcinogenesis in cells from experimental animals and humans.

We have focused our primary attention on two sites of human cancer, i.e., bronchus and esophagus. As noted above, our initial effort was devoted to developing methods to culture and unequivocally identify human epithelial cells. We are now studying the factors controlling growth and differentiation of these normal cells and their malignant counterparts; the ability to culture these normal and malignant cells in chemically defined media is essential for such studies.

Because human bronchial carcinomas and fetal NHBE cells frequently produce polypeptide hormones, e.g., alpha and beta human chorionic gonadotropin (HCG) and gastrin-releasing peptide, we have proposed that these polypeptides may have normal growth promoting functions during fetal development and the regulation of these autocrine growth factors is aberrant in carcinoma cells. Gastrin-releasing peptide (GRP) and its amphibian equivalent, bombesin, stimulate clonal growth of NHBE cells which have approximately 10,000 high affinity membrane receptors for GRP. Although neither alpha nor beta HCG alone is a growth factor, the combination is growth stimulatory, which is consistent with the hypothesis that beta HCG binding to its membrane receptor allows access of alpha HCG to its receptor to trigger the subsequent mitogenic stimulus. These observations suggest that studying the regulation of the genes of these polypeptide hormones in fetal versus adult NHBE cells and in bronchial carcinomas may reveal the molecular mechanism for their control and their possible role in carcinogenesis. Blood-derived serum (BDS) contains both mitogenic and differentiation-inducing factors. Supplementation of the culture medium, LHC-9, with as little as 0.25% fetal bovine BDS results in a decrease in clonal growth rate of NHBE cells; 8% supplementation completely inhibits growth by inducing terminal squamous cell differentiation. Human lung carcinoma lines were also incubated in LHC-9 medium without and with 8% BDS. The results showed that serum toxicity per se is not responsible for the observed inhibition of NHBE cell growth; all 10 carcinoma lines divided significantly more rapidly ($p < 0.05$) in BDS-supplemented medium. Thus, the carcinoma cells have both increased requirements for

BDS mitogens and a greatly reduced ability to respond to factors in BDS that induce the normal cells to undergo squamous differentiation. Since cell planar areas and opposition index increase in direct proportion to the concentration of BDS, these characteristics can be used as a quantitative assay for squamous differentiation-inducing activity. Immunoperoxidase staining for involucrin clearly revealed that NHBE cells exposed to BDS are arranged in a multilayered fashion. The overlying cells are large and strongly involucrin positive, whereas the basal cell sheets are involucrin negative.

Type B transforming growth factor (TGF-B) isolated from human platelets was studied as the serum factor responsible for inducing cell to undergo squamous differentiation. NHBE cells were shown to have high affinity receptors for TGF-B. TGF-B induced the following markers of terminal squamous differentiation in NHBE cells: 1) increase in Ca ionophore induced formation of cross-linked envelopes; 2) increase in extracellular activity of plasminogen activator; 3) irreversible inhibition of DNA synthesis; 4) decrease in clonal growth rate; and 5) increase in cell area. The IgG fraction of anti-TGF-B antiserum prevented both the inhibition of DNA synthesis and the induction of differentiation by either TGF-B or blood-derived serum. Therefore, TGF-B is the primary differentiation-inducing factor in serum for NHBE cells.

TGF-B did not inhibit growth of human lung carcinoma cell lines on Ha-*ras* oncogene transfected NHBE cells in the serum-free monolayer culture system. TGF-B specific receptor assay revealed that the differential effects of TGF-B on NHBE cells and human lung malignant cells are not because of lack of TGF-B receptors on malignant cells. Epinephrine antagonized TGF-B-induced inhibition of DNA synthesis and squamous differentiation of NHBE cells, although epinephrine increased cyclic AMP levels in NHBE cells either in the presence or absence of epinerphrine. Therefore, the action of epinephrine on TGF-B effect appears to be via indirect mechanisms.

Terminal squamous differentiation in the normal bronchial epithelial cells can be induced by blood-derived serum, platelet lysates, TGF-beta suspension in semisolid medium, confluence culture conditions or calcium ions (> 1 mM) and small amounts of serum. However, these inducers of differentiation do not have the same effects in either carcinoma cells or, as to be described later, oncogene-transformed cells which continue to grow and, in some cases, grow at a faster rate. These observations are consistent with the hypothesis that preneoplastic and neoplastic cells are resistent to endogenous and exogenous inducers of terminal differentiation and thus have a selective survival-growth advantage. We are currently identifying these inducers of differentiation of normal human bronchial epithelial (NHBE) cells and studying their mechanisms of action.

The information from the above-mentioned studies is being used in the design of in vitro carcinogenesis experiments in which these inducers of terminal differentiation are being used in a strategy to provide selective advantage of preneoplastic and neoplastic cells.

CARCINOGENESIS STUDIES

Carcinogenesis is a multistage process that can be operationally divided into tumor initiation, promotion, conversion, and progression. Genetic changes, perhaps mutations, are considered to be responsible for tumor initiation and malignant conversion. As will be discussed in a latter section, metabolism of

carcinogens, DNA damage, and DNA repair are considered to be important factors in these stages of carcinogenesis.

Tumor Promotion

Selective clonal expansion of preneoplastic ("initiated") cells is a basic tenet of tumor promotion. Examples of mechanisms that could lead to selective clonal expansion of "initiated" cells compared with normal cells include (a) resistance to either exogenous or endogenous inducers of terminal differentiation, (b) resistance of the preneoplastic and neoplastic cells to cytotoxic products of integrated viral genes, (c) enhanced expression in the preneoplastic cells of either cellular or integrated viral genes whose products stimulate cell division, (d) autocrine production of growth factors, (e) increased sensitivity of the initiated cell to growth factors, and (f) cell surface modifications, both antigenic and functional, that could cause aberrant intercellular communication, recognition, and adhesion. In the previous section, examples of endogenous growth factors and squamous differentiation factors were described.

Tumor promoters are examples of exogenous agents that may induce terminal differentiation in one cell type and stimulate another subpopulation to proliferate. Therefore, the effects of tumor promoters, such as 12-O-tetradecanoyl-phorbol-13-acetate (TPA), teleocidin B, and aplysiatoxin on growth and differentiation of NHBE cells have been investigated. Nanomolar quantities of TPA rapidly inhibit the clonal growth rate of NHBE cells and concomitantly induce terminal squamous differentiation as measured by an increase in cell surface area, progressive stratification of the squamous cells, enhanced plasminogen activator activity, and increased formation of cross-linked envelopes. Teleocidin B, aplysiatoxin, and 2,3,7,8-tetrachlorodibenzodioxin, tumor promoters with markedly different chemical structures from each other and TPA, also cause similar changes. In contrast, ten different human lung carcinoma cell lines were relatively resistant to TPA induction of terminal differentiation. Therefore, TPA may be useful for identifying preneoplastic human cells and for allowing the selective growth of these cells during in vitro carcinogenesis studies.

Tobacco smoke has been shown in experimental animal studies to contain both tumor initiators and promoters. Therefore, we are comparing the effects of tobacco smoke components with those of tumor promoters (e.g., TPA) in cultured human bronchial epithelial cells. In our initial study the effects of cigarette smoke condensate (CSC), two basic fractions (BIa, BIb) of CSC, the ethanol extract weakly acidic fraction (WAe), and the methanol-extracted neutral fraction (NMeoh) on the clonal growth rate, plasminogen activator (PA) activity, cross-linked envelope (CLE) formation, ornithine decarboxylase (ODC) activity, EGF binding, thiol levels, and DNA single strand breaks (SSB's) were investigated. Neither CSC nor any of the fractions were mitogenic over the range 0.01-100 ug per ml. All were growth inhibitory at higher concentrations. The 50% growth inhibitory concentrations (IC50) for CSC, BIa, BIb, WAe, and NMeoh were 10, 10, 10, 3, and 1 ug/ml, respectively. Effects on CLE formation, morphology, PA and ODC activities, epidermal growth factor (EGF) binding, and thiol levels were evaluated using IC50 concentrations. We found that CSC and all fractions tested caused an increased formation of CLE from a baseline of 0.5% in the untreated cells to an increase of 20% induced by NMeoh. A squamous morphological change was observed within one hour after expsoure to NMeoh, WAe, and CSC. The BIa and BIb fractions had little effect. Only NMeoh

increased PA significantly, from 2.5 ± 0.4 to 5.1 ± 0.3 units/mg cellular protein. CSC and the WAe and NMeoh (NMeoh > WAe > CSC) fractions caused a decrease in EGF binding, in each case reaching a maximum effect after a 1-12 hour incubation. At the IC50 neither CSC nor any of the fractions significantly affected intracellular thiol levels. CSC caused significant DNA SSB only at a concentration of 100 ug/ml levels. Neither CSC nor any of the fractions had an effect on ODC activity. Due to the effects of the NMeoh fraction on growth, morphology, EGF binding, and PA activity, we consider it to be the most likely portion of CSC to contain compounds with actions similar to those of the phorbol ester, indole alkaloid and polyacetate tumor promoters.

Acute effects of putative cocarcinogens and tumor promoters were also investigated using cultured human esophageal epithelial cells in serum-free LHC-8 medium and 199 medium containing 10% fetal calf serum. The effects were evaluated on clonal growth rate, cross-linked envelope formation, and the enzymatic activities of ornithine decarboxylase and plasminogen activator. The major findings included: teleocidin B, capsaicin, and cigarette smoke condensate were inhibitory to clonal growth; in contrast to results found in NHBE cells, none of the compounds induce CLE formation under conditions used; TPA induced ODC in medium 199, but inhibited it in LHC-8 media. Capsaicin induced ODC in both media; and TPA and capsaicin each induced plasminogen activator activity in both media. These observations will be utilized in the design of in vitro carcinogenesis studies.

Oncogenes

Activation and/or modification of cellular oncogenes is likely to be important in carcinogenesis. Because the vast majority of the studies utilized an interspecies assay, i.e., transfection of human tumor DNA into mouse NIH 3T3 cells, we are using human epithelial cells as recipients of oncogene DNA transfected into the cells by a modified protoplast fusion method. Transfection of primary human bronchial cultures with plasmids carrying the vHa-ras oncogenic complementary DNA results in the following changes in the epithelial cells: altered cell growth properties, resistance to inducers of squamous differentiation, immortality, progression to anchorage independent growth, and tumorigenicity. The characterization of one of these recombinant cell lines (TBE-1) has established cells from clonal isolation at various stages of development for carcinogenic complementation studies with (1) other oncogenes and (2) chemical and physical carcinogens. TBE-1 has integrated vHa-ras into its genome and expresses transcripts that hybridize to Ha-specific structural gene and vHa-LTR probe DNA. TBE-1 cells express detectable levels of phosphorylated vHa-ras polypeptide, p21.

TBE-1 cultures were tested to determine growth characteristics, response to TPA, the ability to form colonies of anchorage independent cells in soft agar, and tumorigenicity in athymic nude mice. The results indicate that TBE-1 cells (1) are not induced to squamous terminal differentiation by BDS, confluence, or 10^{-7}M TPA, (2) form colonies in soft agar, and (3) produce an autogenous growth factor since population doublings per day (PD/D) increase by a factor of 5.0 when autogenously conditioned medium is used to supplement cell growth at clonal density. The injection of nude mice with TBE-1 cells before selecting an anchorage independent population leads to the development of nodules less than 0.1 cm in size that regress after 14 days and in some animals reappear after 9-12 months. Isozyme phenotype analysis for six human isozyme markers following electrophoretic separation and staining of cell

extracts indicates that TBE-1 cells are human. The morphology of the injected cell nodules resembles an anaplastic carcinoma.

TBE-1SA cells were isolated by selection of anchorage independent cells growing in soft agar cultures of TBE-1 cells. The TBE-1SA cells were characterized for tumorigenicity, karyology, isozyme phenotype, and histocytochemical staining for keratin and beta human chorionic growth hormone (HCG). The progression of this subpopulation is indicated by its ability to form tumors that can grow to > 2cm, do not regress and form metastases to liver, lung, spleen and kidney. Positive histocytochemical staining of TBE-1SA nodules for keratin and immunoprecipitation-gel electrophoresis analysis for cytokeratins confirms the epithelial origin of TBE-1SA cells. Since more than 70% of bronchogenic carcinomas contain detectable levels of HCG, TBE-1SA tumors were stained for beta HCG. The histocytochemical characterization reveals beta HCG product within TBE-1SA tumor cells. The tumorigenicity of TBE-1SA is in contrast with the pretumorigenic phenotype of unselected TBE-1 cell populations that form nodules which regress 14 days after injection. The pretumorigenic TBE-1 cells produced no anchorage independent colonies within the limits of detection (10^{-6}) immediately after focus formation and were expressing vHa-ras gene transcripts and p21 gene product during the period of growth and progression to tumorigenicity of the population.

The karyotypic analysis of TBE-1SA cells and isozyme phenotype of TBE-1SA tumor tissue collected 84 days after transplantation indicate that these cells are human and that the tumor tissue is identifiable as TBE-1SA by the correspondence of isozymes measured from the tumor tissue. The modal distribution of chromosomes is 74-75 for TBE-1SA, with marker chromosomes and extensive chromosomal abnormality. The karyotypic instability of TBE-1 cells was also an early event detectable at the first passage after the isolation of foci, and the karyology of TBE-1SA cells shows the extent of abnormality the bronchial epithelial vHa-ras transfectants have sustained during their progression to tumorigenicity in athymic nude mice. The transfected v-Ha-ras oncogene apparently caused NHBE cells to become immortal and malignant as judged by their continued growth, aneuploidy, and tumorigenicity in athymic nude mice. The mechanisms by which the v-Ha-ras p21 initates this multistage process is unknown. Because aneuploidy was an early observation, we propose that the mutant p21 causes biochemical changes that interfere with mitosis and cause chromosomal rearrangements, including loss of specific chromosomes containing cancer-suppressor genes that allow the emergence of the malignant cells from the population of less malignant cells in response to the selective pressures provided by BDS, high-density cell cultures, and growth in semisolid medium. These selection pressures are potent inducers of terminal squamous differentiation of NHBE cells, but human lung carcinoma cell lines are relatively resistant and continue to grow. It is of interest that although TPA was not used as a selective pressure in their isolation, the transformed cells nonetheless acquired a resistance to induction of differentiation by TPA. This observaton implies a more generalized defect in the differentiation program of these transformed cells.

The data noted above are consistant with the hypothesis that preneoplastic and neoplastic human bronchial epithelial cells have an imbalance in their growth and differentiation programs. Such an imbalance would provide these cells with a selective growth expansion advantage over the normal epithelial cells. In vitro carcinogenesis studies by other investigators (e.g., Yuspa, Scott, etc.), using cells from experimental animals, have also provided data supporting this hypothesis. Defects in control of cellular differentiation have been associated with the initiation phase of carcinogenesis in mouse epidermal cells and 3T3 T proadipocytes.

The raf oncogene/proto-oncogene is an evolutionarily ancient member of the largest family of oncogenes, the src family. These genes, many of which have been shown to possess a tyrosine kinase activity, are found associated with the cell plasma membrane and are believed to be components of the cell's receptor systems. The recognition, isolation, and characterization of these potentially deleterious cellular genes should lead to an understanding of the biochemical functions which drive a cell to cancerous growth. Many proto-oncogenes have been highly conserved through evolution since the appearance of metazoan organisms; hence, the belief that their role in normal cellular development and differentiation has transgressed time unaltered. Evolutionarily significant homologs of the raf oncogene/proto-oncogene have been isolated from Drosophila and Saccharomyces and are being characterized so as to define their role in the biochemical pathways controlling cellular proliferation. The Drosophila homology has been shown to be essential for embryonic development and probably is one of four genes localized to the 2F region of the X chromosome. Direct manipulations of this sequence are now possible in the Drosophila and will soon be possible in yeast. As an extension of these studies two new human proto-oncogenes, distantly related to the raf sequence, have been recognized. One of these proto-oncogenes, c-pks-1, has been localized to the short arm of the X chromosome (the other is on the short arm of chromosome 7). Although much of the accumulated experimental data deal with cancer, nonmalignant proliferative syndromes which may progress to frank neoplasias need to be the subject of more investigations. The use of proto-oncogenes to transmit the signal from mitogen to cellular proliferation suggests these genes will be responsible for hyperplastic as well as neoplastic disorders. The constitutively high expression of c-pks-1 in the peripheral blood mononuclear cells obtained from an individual with the lymphoproliferative disease, angioimmunoblastic lymphadenopathy, compared with the lack of expression in normal cells strongly implicates this gene as contributory to the pathology. The recent finding that Westcot-Aldridge syndrome and familiar immunodeficiency diseases are the consequence of defects residing on the X chromosome has led to the examination of c-pks-1 in these individuals. The mechanism of activation, or inactivation, of the c-pks-1 sequence in these cases is being ascertained.

Both murine and human c-raf-1 are activated in both mitogen stimulated lymphoid cells and in those cells derived from the MRL lpr/lpr autoimmune mouse or human systemic lupus eyrthematosus and associated imflammatory diseases. These cells also express elevated amounts of c-myb RNA. In addition, all small cell lung carcinoma (SCLC) cell lines express $\geq$ 10-fold more c-raf-1 RNA than normal bronchial epithelial cells, and the metastases contain 10 to 25 times more c-raf-1 than the SCLC cell lines. Employing fluorescence conjuated monoclonal antibodies and the FACS, the surface characteristics of various SCLC cell lines were determined. Two early monomyelocyte markers were present on the SCLC cells.

Although in the above examples of possible raf proto-oncogene involvement in excessive cellular proliferation, alterations of the coding sequences seem unlikely, recent data suggest that in both of the pancreatic carcinoma DNA examined truncations of the 5'end of the gene had taken place. This would be expected to functionally change controlling elements of this protein which maintain the ordered use of this presumed receptor.

Physical Carcinogenesis Studies

Epidemiological studies have established that exposure to asbestos fibers is the primary cause of mesothelioma in the industrialized world. Because the latency period for this disease averages 40 years and because there has been a marked increase in the use of asbestos during and since World War II, an epidemic of mesothelioma has been predicted for the latter part of this century. Carcinogenesis studies with animals have shown that mesothelioma can be caused by intrapleural or intraperitoneal injections of asbestos. However, the long-term effects of asbestos fibers on human mesothelial cells in culture have not been reported previously. To study this important problem, methods to culture replicative normal mesothelial cells from adult human donors have been developed. The cells contain keratin and hyaluronic acid-mucin, exhibit long, branched microvilli, and retain the normal human karyotype to senescence. The mesothelial cells are 10 and 100 times more sensitive to the cytotoxicity of asbestos fibers than are bronchial epithelial or fibroblastic cells, respectively, from normal adult humans. Exposure of the mesothelial cells to amosite asbestos causes chromosomal rearrangements, including dicentrics. These aneuploid mesothelial cells have an extended population doubling potential of more than 35 divisions beyond the culture life span (30 doublings) of the control cells. One possible effect of asbestos exposure is that rearrangments of chromosomes may result in increased transcription of specific oncogenes or growth factors. We have detected markedly increased production of TGF-B by a mesothelioma cell line; additional studies on growth factor requirements of normal mesothelial cells have determined that TGF-B is a potent mitogen for these cells.

Another important problem in human carcinogenesis concerns the mechanism responsible for the cocarcinogenic effect of asbestos in enhancing the tumorigenicity of tobacco smoke in the bronchial epithelium. We and our coworkers have initiated investigations to define the effects of asbestos on cultured bronchial epithelial tissues and cells. The differential cytotoxic activity of various asbestos and glass fibers was estimated by measuring the inhibition of epithelial cell growth as a function of fiber concentration. The data show that various fiber types have different effects on human bronchial epithelial cells. Chrysotile was extremely toxic; amosite and crocidolite were less toxic; glass fibers were only mildly toxic. For comparison, human bronchial fibroblastic cells were also exposed to fibers and were found to be markedly more resistant (more than ten-fold) than the epithelial cells to all of the types of asbestos tested.

Monolayers of bronchial epithelial cells and of mesothelial cells were used to investigate the effect of amosite asbestos at the cellular level. With scanning electron microscopy and high voltage electron microscopy, amosite fibers ingested by human bronchial epithelial cells can be seen. In contrast to macrophages, the fibers penetrate the surface of epithelial cells without the development of filopodia. While macrophages and mesothelial cells seem to phagocytose fibers along both the long and short axes, epithelial cells seem to take up fibers along the short axis with only a membrane sleeve surrounding each fiber. Examination by scanning high voltage and transmission electron microscopy with associated energy dispersive X-ray spectra clearly revealed that short fibers (< 12m) are taken up quickly by the cells. Asbestos is present within the cells by 2 hours after exposure; and by 28 hours, many fibers are found in the cytoplasm and occasionally in the nucleus. Acid phosphatase staining of mesothelial cells after phagocytosing asbestos fibers reveals negligible release of lysosomal contents in response to the fibers. Cells exposed to amosite and

arrested in metaphase have been found to have numbers of fibers adherant to the chromosomes. Several studies (electron paramagnetic response, the use of free radical scavengers, and measurement of DNA damage) have all suggested that oxygen radicals are not important for the cytopathic effect of asbestos fibers on mesothelial cells. It is possible that asbestos perturbs the cytoskeleton or mechanically disturbs metaphase chromosomes.

Asbestos fibers induce abnormal cell growth. Addition of amosite asbestos (10, 100, or 1,000 ug/ml) to human respiratory mucosa in explant culture causes numerous focal lesions including squamous metaplasia and dysplasia. When examined by scanning electron microscopy, the epithelial lesions appear as focal elevations of nonciliated cells.

Cytopathological aberrations of the bronchial epithelial cells are manifested by cellular polymorphism and variation in nuclear size. Since it is important to know whether there are asbestos fibers in the cytoplasm of cells involved in the lesions, studies are now being done using X-ray microanalysis in combination with transmission electron microscopy including high voltage electron microscopy. These studies are being extended to determine the progression of these lesions and eventually their malignant potential.

Epidemiological studies have shown that inhalation of nickel compounds enhances the risk for human respiratory cancer. Cultures of normal human bronchial epithelial cells were continuously exposed to a dose (5-20 ug/ml) of Ni_2SO_4 that reduced their colony-forming efficiency 30-80%. After 40 days of incubation, the cultures consist of large, squamous cells; mitotic cells are very rare. The cells are then maintained in growth medium without nickel. After 40-75 total days of incubation, colonies of mitotic cells appear at a rate of 1 colony per 100,000 cells at risk; no colonies appear in control cultures or in cultures exposed to < 5 ug of nickel/ml for 90 days. Twelve cultures isolated from five experiments have been expanded into mass cultures. Most of the cell lines have an increased population doubling potental (> 50 divisions). Some have a reduced response to squamous (terminal) differentiation-inducing signals, whereas others have lost growth factor requirements for clonal growth. Aneuploidy and marker chromosomes have also been noted. However, none of these Ni-altered clones of bronchial epithelial cells is anchorage independent and they do not produce tumors upon injection into athymic nude mice. Experiments are planned to ascertain if these abnormal cells will progress to tumorigenicity after either re-exposure to carcinogens or transfection with oncogenes.

Methylation

The pattern of 5-methylcytosine residues in mammalian DNA has recently been found to be crucial to the control of genetic expression. Decreases in DNA 5-methylcytosine content are known to alter the level of differentiation of cells in culture. Thus, changes in DNA 5-methylcytosine patterns may be critical carcinogenesis. Human tumor DNA is being probed for DNA methylation pattern alterations in selective DNA sequences and genes. Since chemical carcinogens have been shown to decrease genomic 5-methylcytosine levels in BALB-3T3 cells, DNA from carcinogen-treated human epithelial cells is also being probed for changes in 5-methylcytosine patterns. New methods to assess 5-methylcytosine content in non-dividing differentiated human cells have been developed. Previously, the determination of genomic 5-methylcytosine levels required the labeling of DNA in dividing cells with tritiated-uridine. Limitations in

epithelial cell numbers required toxic levels of tritium in order to sufficiently label the DNA for 5-methylcytosine measurements. We have now developed a new method which is both sensitive and does not require active DNA synthesis and cell division. DNA from any source can be enzymatically digested to nucleotides and labeled with ^{32}P. The labeled nucleotides are then separated by TLC and the ratio of 5-methylcytidine to the total cytidine and 5-methylcytidine determined. The genomic level of 5-methylcytidine has been measured for the first time in normal human bronchial epithelial and pleural mesothelial cells. This highly sensitive ^{32}P post-labeling method not only enables the above-described chemical carcinogenesis studies to be performed on human epithelial cells but also allows for monitoring of genomic as well as specific gene levels of 5-methylcytosine in tumors, tissues, and cell types from human and animal sources. Thus, changes in 5-methylcytosine levels in specific genes, including oncogenes, during differentiation and carcinogenesis and during the normal aging process in vivo can now be followed.

Somatic Cell Genetics

Genetic changes related to carcinogenesis are being studied using hybrids of human lung carcinoma cells with NHBE cells. We are interested in determining if the malignant phenotype and immortality of human bronchial epithelial cells are recessive or dominant. For these experiments, two bronchial carcinoma cell lines (ouabain-resistant and HPRT minus) have been produced to be used as universal recipients. A limited population doubling potential (mortality) is a dominant genetic trait in tumor-normal hybrid cells. Other hybrid cell lines using lung carcinoma cell lines and H-ras transfected bronchial epithelial cells as parents have been isolated and are being characterized for doubling potential, karyotype, and tumorigenicity in athymic nude mice.

INTERMEDIATE FILAMENTS IN NORMAL AND CARCINOMA CELLS

Cancers of the human esophagus and lung represent major causes of death in certain populations of people throughout the world. In focusing our efforts on these two organ systems, we have characterized the pattern of expression of the main markers of epithelial differentiation, namely keratins, involucrin, and cross-linked envelopes, during the course of embryonic development, post-natal maturation, and/or in neoplasia. The pattern of expression was found to be dependent on cell type, the stage of differentiation and/or development, and the extrinsic environment of the cell. Distinctive qualitative and quantitative differences in the spectrum of keratin proteins are found in the carcinomas compared to their nontransformed counterparts. Analysis of keratin protein patterns is a useful adjunct in defining the type of tumor present. Moreover, assessment of cross-linked envelope-forming capabilities and the presence of involucrin serves as specific markers for squamous differentiation and the extent of envelope formation and involucrin staining correlates well with the degree of squamous differentiation in the tumor with more well-differentiated squamous carcinomas forming more cross-linked envelopes or possessing involucrin. We have established human esophageal and lung carcinoma cell lines in cell culture to evaluate if their properties in vitro faithfully manifest those of the original tumor, thereby representing useful models of carcinogenesis in vitro. Moreover, we have compared the growth and differentiated properties of these carcinoma cells to their non-transformed counterparts. Numerous morphological and biochemical differences are observed between normal and malignant epithelial cells in culture. Significant changes in the array of keratins and in the proportions of cells making cross-linked envelopes were found. The

results we obtained parallel findings with tumor masses indicating that the tumor cells in cell culture continue to maintain a program of gene expression reflective of that of the original tumor.

METABOLISM OF CHEMICAL CARCINOGENS AND FORMATION OF CARCINOGEN-DNA ADDUCTS

The earliest events in the multistage process of chemical carcinogenesis are thought to include (1) exposure to the carcinogen; (2) transport of the carcinogen to the target cell; (3) activation to its ultimate carcinogenic metabolite, if the agent is a procarcinogen; and (4) DNA damage leading to an inherited change. Therefore, one important use of cultured human tissues has been in the investigation of the metabolism of chemical carcinogens because (1) many environmental carcinogens require metabolic activation to exert their oncogenic effects; (2) the metabolic balance between carcinogen activation and deactivation may, in part, determine a person's oncogenic susceptibility; and (3) knowledge of the comparative metabolism of chemical carcinogens among animal species will aid efforts to extrapolate data on carcinogenesis from experimental animals to humans. We and our coworkers have systematically examined the metabolism of procarcinogens of several chemical classes which are considered to be important in the etiology of human cancer. Procarcinogens of several chemical classes can be activated enzymatically to electrophilic reactants that bind covalently to DNA in cultured human tissues. The studies of activation and deactivation of representative procarcinogens have revealed that the metabolic pathways and the predominant adducts formed with DNA are generally similar between humans and experimental animals. Wide quantitative interindividual differences (50- to 150-fold) are found in humans and other outbred animal species. When the metabolic capabilities of specimens from different levels of biological organization are compared, the profile of benzo[a]pyrene metabolites is similar in cultured tissues and cells, but subcellular fractions, e.g., microsomes, produce a qualitative and quantitative aberrant pattern.

The metabolism of benzo[a]pyrene was studied in both epithelial and fibroblastic cells initiated from the same bronchus specimens. The total metabolism of benzo[a]pyrene and binding of its ultimate carcinogenic metabolite is three-fold higher in the epithelial than in the fibroblast cells. No qualitative differences in the metabolic profile of benzo[a]pyrene between the explant culture and the epithelial cell cultures were observed.

To test the interactive effects of cell types in the metabolic activation of carcinogens and to further assess interindividual differences among people, human tissue- and cell-mediated mutagenesis assays have been developed. The fact that terminally differentiated cells, such as pulmonary alveolar macrophages, can activate benzo[a]pyrene and mediate an increase in frequencies of mutations and sister chromatid exchanges in cocultivated "detector" cell populations (i.e., Chinese hamster V79 cells) suggests that nontarget cells of chemical carcinogens may play an important role in the activation of environmental carcinogens.

Fecapentaenes have been identified by Wilkins, Bruce and others as the major contributors to the mutagenicity found in human feces. Because these direct-acting mutagens in Salmonella bacteria are both potential carcinogens and may play a role in the etiology of human colonic carcinoma, we have initiated a series of invesitgations to assess their pathobiological effects and mechanism of action. Fecapentaene-12 has been shown in human fibroblasts to (a) cause

6-thioguanine-resistant mutations and higher frequencies were found in xeroderma pigmentosum fibroblasts when compared to normal cells; (b) cause single strand DNA breaks; (c) enhance the frequency of sister chromatid exchanges; and (d) cause unscheduled DNA synthesis. In a bacterial plasmid assay, fecapentaene-12 causes (a) mutations, (b) DNA-DNA cross-links, and (c) DNA fragmentation. Finally, fecapentaene-12 transforms mouse Balb 3T3 cells. We are currently examining the effects of fecapentaene-12 in human colonic cells, identifying the DNA adducts and preparing antibodies to these adducts for use in immunoassays to search for adducts in human populations.

The extrapolation of data from studies of N-nitrosamine carcinogenesis between experimental animals and humans is a pressing problem. Abundant evidence of N-nitrosamine carcinogenesis from both in vitro and in vivo studies using experimental animals has accumulated. Although N-nitrosamines are widespread pollutants, the carcinogenicity of these chemicals in humans has been difficult to prove by epidemiological studies. In vitro studies comparing pathobiological responses of N-nitrosamines in humans and experimental animals offer an approach to solve this problem at least at the cellular and tissue levels of biological organization. N-nitrosamines can be metabolized by cultured human epithelial tissues and cells. Quantitative differences in metabolism and alkylation of DNA are found among humans, among various organs within an individual, and among adult versus fetal tissues. Whether these differences are sufficient to influence an individual's cancer risk and organ site is as yet unknown.

DNA REPAIR

Although DNA repair has been extensively studied in human fibroblasts, lymphoid cells, and neoplastic cells, little information is available concerning DNA repair in normal human epithelial cells. Using the methodology to culture human bronchial epithelial and fibroblastic cells developed in our laboratory, we have initiated studies to investigate DNA damage and repair caused by chemical and physical carcinogens as examined by alkaline elution methodology, BND cellulose chromatography, unscheduled DNA synthesis, and high pressure liquid chromatographic analysis of the formation and removal of carcinogen-DNA adducts. As we reported last year, human bronchial epithelial cells repair single-strand breaks in DNA damaged by X-radiation, UV-radiation, chromate, polynuclear aromatic hydrocarbons, formaldehyde, or N-nitrosamines at rates similar to bronchial fibroblasts.

Inducible increase in the resistance to certain N-nitroso compounds is clearly established in *E. coli* by Cairns and coworkers. The biochemical basis for this adaptive response involved increased activities of O6-methylguanine-DNA methyltransferase (O6-MT) and 3-methyladenine-DNA glycosylase II. Because the lung is a target tissue for inhaled N-nitroso compounds in tobacco smoke and environmental pollutants and adaptation, i.e., increased DNA repair capacity following repeated exposures to low doses of alkylating agents, reduce the genotoxicity of low doses of these carcinogens, we have investigated the adaptive response in lung cells. Normal human bronchial epitheial cells cultured in serum free medium were exposed to low doses of N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) to examine whether increased cellular resistance and increased activity of the DNA repair enzyme O6-methylguanine-DNA methyltransferase could be induced. After treatment with single doses of MNNG a dose dependent decrease in O6-methylguanine-DNA methyltransferase activity was observed, as expected for this unique repair system. The activity recovered to the starting level in about 24 h when a dose that consumed approximately 65% of the enzyme activity

(0.2 ug/ml) was given, but did not exceed the activity in the untreated control. Futhermore, treatment every 6 h for 4-5 days with non-toxic concentrations of MNNG (0.04-0.12 ug/ml) did not increase O6-methylguanine-DNA methyltransferase activity; neither was cell survival following a range of challenge doses significnatly increased. Our data suggest that human bronchial epithelial cells do not adapt to MNNG.

During metabolic activation, N-nitrosodimethylamine yields equal molar quantities of methyl carbonium ions and formaldehyde. Both of these metabolites can react with nucleophilic sites in cellular macromolecules, carbonium ions by alkylation, and aldehydes via formation of unstable alkyl-ol derivatives preferably with amine groups (R-HN-CHOH-R_1). The monomethylol derivatives of formaldehyde can form intermediary labile products that by secondary reactions can yield stable methylene bridges between macromolecules. Although the alkylating metabolites of N-nitrosamines and their cytotoxic, mutagenic, and carcinogenic effects have been extensively studied, the possible contribution of other metabolites, especially aldehydes, has not received much attention. We have investigated the effect of formaldehyde on the repair of X-ray-induced single-strand breaks. Human bronchial cells were exposed to X-rays and then incubated with or without the presence of formaldehyde, and the repair of DNA single-strand breaks was measured. The presence of formaldehyde significantly inhibits the repair of the X-ray-induced single-strand breaks correlating with the potentiation of cytotoxicity in human cells and mutation frequency in Chinese hamster V79 cells by combinations of the agents. Formaldehyde, a common environmental pollutant and metabolite of carcinogenic N-nitrosamines, also inhibits repair of O^6-methylguanine, decreases O^6-alkylguanine alkyltransferase activity, is mutagenic at high concentrations (> 100 uM), and potentiates the cytotoxicity and mutagenicity of the methylating agent, N-methyl-N-nitrosourea, in normal human cells. Exposure to formaldehyde may lead to the dual genotoxic mechanism of both directly damaging DNA, i.e., formation of DNA-protein cross-links and single-strand DNA breaks, and inhibiting repair of mutagenic and carcinogenic DNA lesions caused by alkylating agents and physical carcinogens.

Aldehydes and peroxides are of particular interest because of their presence in the environement, e.g., tobacco smoke, and their potential role in tumor promotion. Therefore, we are currently studying the effects of aldehydes (acrolein, formaldehyde, and acetaldehyde) found in tobacco smoke and aldehydes as well as peroxides generated by membrane damage on the growth and differentiation of normal human bronchial epithelial cells. In the initial study, cells were exposed to formaldehyde (Fmd), acetaldehyde (Act), benzoyl peroxide (BPo), or hydrogen peroxide (HPo). The effect of each agent on the following paramters was measured: (a) clonal growth rate, (b) squamous differentiation, (c) DNA damage, (d) ornithine decarboxylase (ODC) activity, (e) nucleic acid synthesis, (f) aryl hydrocarbon hydroxylase (AHH) activity, and (g) arachidonic acid (ArA) and choline (Ch) release. None of these were mitogenic and their effects were assessed at concentrations which reduced growth rate (population doublings per day) to 50% of control (ID50). The ID50 concentrations for the 6 hr exposure were found to be 0.065 mM BPo, 0.21 mM Fmd, 1.2 mM HPo, and 30 mM Act. BPo-exposed cells were smaller than controls (median cell planar area, 620 u2 versus 1150 u2), and Act-exposed cells were larger than controls (median cell planar area, 3200 u2). All agents increased the formation of cross-linked envelopes and depressed RNA synthesis more than DNA synthesis. HPo caused DNA single strand breaks, while Fmd and BPo caused detectble amounts of both single strand breaks and DNA-protein cross-links. Other effects included incrased ArA and Ch release due to HPo. When compared to formaldehyde and

acetaldehyde, lipid peroxidation aldehydes of the 4-hydroxy-a-B-unsaturated type were more cytotoxic and decreased the intracellular thiol content in cultured human bronchial fibroblasts after treament with micromolar concentrations. The unsaturated aldehydes including acrolein also markedly inhibited the DNA repair enzyme, O6-mehytlguanine-DNA methyltransferase, known to have a cysteine residue in its active site, but had no effect on the activity of uracil-DNA glycosylase. Our results indicate that reactive aldehydes of either exogeneous or endogenous origin have direct cytotoxic effects and may also make cells more susceptible to other toxic chemicals due to an impairment in cellular defense mechanisms, e.g., DNA repair and detoxification by systems requiring glutathione.

INTERACTIVE EFFECTS BETWEEN CHEMICAL CARCINOGENS AND HEPATITIS B VIRUS IN LIVER CARCINOGENESIS

Liver cancer incidence is high in areas with both food contamination by carcinogens, such as aflatoxin B_1, a liver carcinogen in experimental animals, and a high incidence of chronic active viral hepatitis. Due to the insensitivity of epidemiological methods, the role of single agents or combinations of agents is uncertain. Our long-term goal is to study the interactive effects of hepatitis B virus and chemical carcinogens, such as aflatoxin B_1, in the malignant transformation of cultured human hepatocytes.

In order to assess the role of chemical carcinogens, e.g., aflatoxin B_1, in human liver carcinogenesis, we have initiated several projects with coworkers at the Cancer Institute, Beijing, People's Republic of China. For example, the metabolic activation of aflatoxin B_1 and other carcinogens, such as N-nitrosodimethylamine and benzo[a]pyrene, have been investigated using cultured human fetal liver explants. One major aflatoxin B_1-DNA adduct is formed by addition of aflatoxin B_1-2,3-oxide to the 7-position of guanine. This reaction product is unstable, and the imidazole ring of the guanine will open to stabilize the molecule. The major aflatoxin B_1-DNA adduct is similar to the one formed in fetal human liver explants and in rat liver in vivo, an organ susceptible to the carcinogenic action of aflatoxin B_1.

In a second related project, we collected urine samples in Murang'a district, Kenya, for analysis of aflatoxin B_1-guanine, a "nucleic acid repair product." It has previously been shown that food samples collected in this district are known to be contaminated with aflatoxin B_1, and a positive correlation exists between the dietary intake of aflatoxin B_1 and the incidence of liver cancer. The urine samples collected at the outpatient clinic of Murang'a district hospital were concentrated on C_{18}Sep-Pak columns, and aflatoxin B_1-guanine was isolated by high pressure liquid chromatography in two different systems. Eleven of 126 samples had a detectable level of a compound whose synchronous fluorescence spectrum was identical to chemically synthesized aflatoxin B_1-guanine. The spectrum did not show any bathochromic shift when the pH was made alkaline. These results are an indication of interactions between the ultimate carcinogenic form of aflatoxin B_1 and cellular nucleic acids in vivo and further support the hypothesis that aflatoxin B_1 may play an important role in the etiology of human liver cancer.

The role of hepatitis B virus (HBV) in human cancer is being investigated by i) developing recombinant human cell ines that carry and express HBV genes providing in vitro models to study the interaction between HBV and human cells; and ii) HBV risk-group patients lymphocytes and lymph node tissue nucleic acids

are being analyzed for the presence and molecular structure of HBV. The development of a recombinant human epithelial cell line (GTC2) that contains only the HBV core antigen gene (HBc) provides a model system to study the regulation and expression of an HBV gene required for virus replication. The discovery that HBc gene expression is cytopathologic when expression reaches maximal levels, and the observation that 5-methylcytosine and nutritional factors regulate the expression of the HBc gene indicates that the role HBV infection plays in liver carcinogenesis may include the promotional effect of increased cell division following the destruction of liver cells after HBc induction in vivo. HBc gene expression in human epithleial cells is controlled by the methylation of a Hpa II site located 280 base pairs upstream from the structural gene, and induction following demethylation of this site is dependent upon the growth conditons both for GTC2 and a hepatocellular carcinoma cell line carrying HBV (PLC/PRF/5). The detection of replicative forms of HBV in lymphocytes from chronic active hepatitis (CAH) and aquired immunodeficiency syndrome disease (AIDS) and the stimulation of HBc gene expression when GTC2 or PLC/PRF/5 cells are treated with 100 u of alpha-interferon (a-IFN) suggests that HBV may have a cytolytic effect during the infection of lymphocytes that is important to the immunological abnormalities frequently associated with HBV infection.

BIOCHEMICAL AND MOLECULAR EPIDEMIOLOGY

The primary goal of biochemical and molecular epidemiology is to identify individuals at high cancer risk by obtaining pathobiological evidence of (1) high exposure of target cells to carcinogens and/or (2) increased host susceptibility due to inherited or acquired factors. Laboratory methods have been developed recently to be used in combination with analytical epidemiology to identify individuals at high cancer risk. These methods include (1) techniques to assess specific host susceptibility factors; (2) assays that detect carcinogens in human tissues, cells, and fluids; (3) cellular assays to measure pathobiological evidence of exposure to carcinogens; and (4) methods to measure early biochemical and molecular responses to carcinogens.

Currently available techniques exist that would allow the utilization of biochemical and molecular measures to better characterize exposure to carcinogens, to serve as intermediate end points on the path to malignancy, to identify measures which halt or reverse this process, and to investigate the mechanisms of human carcinogenesis. Included in these investigations would be the following: (1) efforts to evaluate body burden of chemical carcinogens in studies of occupational and general environmental cancer risk factors; (2) sophisticated analyses of air, water, and biological specimens for carcinogenic and mutagenic substances in conjunction with specific analytical studies; (3) search for evidence of viral infection including viral segments or oncogenes in the DNA of individuals at high risk of cancers that may be associated with infectious agents or heritable states; (4) evaluation of disturbances in immune function as they may relate to malignancies, particularly those of the hematopoietic system; (5) investigation of the relationship between micronutrients and a variety of epithelial cancers; and (6) determination of the relationship between macronutrients, including dietary fat and subsequent hormonal changes, to subsequent risk of breast, endometrial, and perhaps colon cancer.

Carcinogen-DNA Adducts and Human Antibodies to Adducts

Our investigations of carcinogen metabolism in cultured human cells revealed that the major carcinogen-DNA adducts formed are identical to those found in experimental animals in which the chemical is carcinogenic. This important finding has encouraged us and others to search for adducts and antibodies to adducts in people exposed to environmental carcinogens. For example, roofers, coke oven workers, and aluminum plant workers as well as cigarette smokers are exposed to high levels of polynuclear aromatic hydrocarbons, including benzo-[a]pyrene (BP). We are currently comparing two very sensitive methods to detect BP-DNA. Both of the methods, USERIA (ultrasensitive enzymatic radioimmunoassay) using rabbit-produced polyclonal antibody and a biophysical method, synchronous fluorescence spectrophotometry, appear to detect about 1 adduct per 1×10^8 nucleotides. Quantitation with both of the methods is possible. USERIA is most sensitive toward BP-DNA; for the synchronous fluorescence spectrophotometry, DNA has to be acid-hydrolyzed to release BP moieties as tetrols. Both qualitative and quantitative results from preliminary experiments comparing the methods have been promising. Another type of evidence of former or current existence of BP-DNA adducts comes from the presence of antibodies toward an epitope on BP-DNA in serum from people exposed to BP. For example, these antibodies have been detected by the ELISA technique from coke oven workers.

Aflatoxin B_1 (AFB)-contaminated food is the main source of AFB exposure. Both USERIA and synchronous fluorescence spectrophotometry are currently being applied in our laboratory to detect AFB-DNA. Synchronous fluorescence spectrophotometry (SFS) can be used to measure fematole concentrations of aflatoxins and their metabolites and DNA adducts. Computer-assisted analysis of highly specific spectra of these agents obtained by SFS can be displayed as 3-dimensional contour maps. Individual agents in mixtures, e.g., aflatoxin B1 and M1 can be identified by 4th derivative spectral analysis. This physical method should complement immunological and other methods to measure aflatoxin B1 and its metabolites and nucleotide adducts in body fluids and DNA adducts in tissues from people exposed to this carcinogen.

With none of these methods have all of the persons among a certain exposed group been positive. The significance of this interindividual difference is as yet unknown. In addition, the formation of carcinogen-DNA adducts may represent only the initial stages of carcinogenesis and measures of later stages, i.e., tumor promotion and progression, are also needed to predict an individual's cancer risk.

The use of antibodies in sensitive immunoassay provides the means of detecting carcinogens adducted to DNA. Sera obtained from individuals who had high exposure to certain chemical carcinogens by virture of their occupation were studied for antibodies to the benzo(a)pyrene DNA adducts. Serum samples were also obtained from different test goups with individuals who were exposed to benzo(a)pyrene by ciagrette smoke. A third group of patients who were studied were those who had tumors that had been linked to cigarette smoking. A total of 541 sera have been tested. Antibodies against the BP-DNA epitope(s) were detected in serum samples of each of these test groups. The percentage of sera-positive individuals was the highest in those who had exposure to benzo(a)pyrene by virture of their occupation. These were the coke oven workers from the USA and Norway where the percentage of positivity ranged from 27 to 34%. A similar number of individuals with lung cancer had antibodies to BP-DNA epitope(s). Sero-positive persons were also identified in non-cancer donors. The highest

levels of antibody were found in those who had the highest levels of exposure (occupational) to benzo(a)pyrene.

Monoclonal antibodies to aflatoxin (AFB) DNA adducts were produced in this laboratory or obtained from commercial sources. These antibodies were used to study and detect the various metabolites of this compound adducted to DNA. Different patterns of reactivity were found with different monoclonal antibodies suggesting that different metabolites did not share common epitopes. Sera were obtained from individuals exposed by virture of diet to high levels of AFB or from individuals where the AFB is controlled at relatively low levels. The majority of the individuals studied appeared to have antibodies to the AFB epitope(s) at least at low levels to AFB. Those individuals with the highest antibody titers were generally those from the areas with high exposure to this carcinogen.

Xenobiotic Polymorphisms

Phenotyping of animal models and man for their xenobiotic metabolizing capabilities has, in recent years, been undertaken in the interests of predicting carcinogenic susceptibilities. The metabolism of exogenous agents is known to be genetically dependent and selective in vivo xenobiotic metabolic routes appear to be accessible to evaluation by the use of nontoxic doses of certain drugs. The determination of the rate of selective enzymatic modifications of test agents may thus provide a suggestion as to how susceptible an individual may be to the oncogenic potential of carcinogens activated by similar metabolic routes. Several agents including debrisoquine (DBQ), S-mephenytoin (SMPH), S-carboxymethyl-L-cysteine (SCMC), and sulfamethazine have been shown to be metabolized by enzymatic routes governed by separate genetic loci. We have initiated both an animal model study and clinical investigations. Since the primate colony from which these monkeys will be sampled has been and is presently involved in ongoing chemical carcinogenesis experiments, the results of the metabolic phenotyping can be compared to the susceptibilities of the monkeys to carcinogenesis.

Preliminary results of phenotyping three monkeys from each of three species, including rhesus, cynomolgus, and African green monkeys, are available. All of the monkeys tested rapidly metabolized DBQ, SMPH, and SCMC. The metabolic ratio (concentration of parent drug to metabolite) was observed to range from 0.02 to 0.6 for DBQ, which is well below that reported for man. No differences between species are observed with the minimal sample size of three monkeys per group. MPH metabolism is extensive in these monkeys as well. From 6 to 52% of the administered dose of MPH is excreted as 4O H-MPH in 24 hours, which is greater than that reported for man. There are no significant differences in the rate of MPH hydroxylation between species.

The rate of sulfoxidation of SCMC is also high in these monkeys. The metabolic ratio ranged between 0.8 and 3.8, but the rhesus and cynomolgus monkeys metabolize SCMC significantly ($p < 0.10$) faster than the African green monkeys. The average SCMC sulfoxidation index per species is 1.4 $\pm$ 0.3, 1.2 $\pm$ 0.3, and 2.7 $\pm$ 0.9 for rhesus, cynomolgus, and African greens, respectively. These rates are more rapid than that reported for man. Rhesus monkeys are significantly ($p < 0.01$) slower N-acetylators of sulfamethazine than African green monkeys, while that of the cynomolgus monkeys fell between the former two species. The average rate of N-acetylsulfamethazine formation is 51 $\pm$ 4%, 65 $\pm$ 12%, and 76 $\pm$ 9% for rhesus, cynomolgus, and African green monkeys, respectively. This rate

of sulfamethazine acetylation in rhesus monkeys compares well with previously reported values. These rates also compare well with the human data, classifying the rhesus as poor N-acetylators, the African greens as extensive N-acetylators, and individual cynomolgus monkeys in both categories.

The rapid rate of enzymatic hydroxylation observed in these monkeys for DBQ and MPH would suggest that these monkeys would be susceptible to carcinogenesis upon exposure to aromatic hydrocarbon carcinogens. AFB and methylazoxymethanol-acetate have been shown by Drs. Adamson and Sieber to produce tumors in these species. However, several other carcinogens, including benzo[a]pyrene, 3-methylcholanthrene, and cigarette smoke condensate did not induce tumors. Thus, there may be some correlation between the metabolic phenotype and chemical carcinogenesis susceptibility, but the data presently available are insufficient to draw firm conclusions.

In collaboration with Dr. Jeff Idle, St. Mary's Hospital, London, we are using DBQ to phenotype lung cancer patients, their relatives, and individuals in various control groups. The extensive and poor metabolism phenotypes are being studied by measuring oncogene expression and presence of carcinogen-DNA adducts in tissues and cells, antibodies to these adducts, urinary levels of polycyclic aromatic hydrocarbons and carcinogen-guanine adducts, and DNA polymorphisms.

Human T-cell Leukemia Virus

Studies were carried out to examine the cell surface markers of cells infected with and producing the human T-cell lymphoma virus (HTLV). HLA typing was performed on these cells. The virus was transferred to human umbilical cord blood lymphocytes by coculture.
Cell lines established from patients with the adult T-cell leukemias and lymphomas express more than the expected two alloantigens controlled by the HLA-A or HLA-B locus. Alloantisera detecting these altered determinants are confined to the HLA-AW19 cross-reactive group and the HLA-B5 cross-reactive group. A monoclonal antibody detecting a polymorphic epitope on HLA alloantigens of these two cross-reactive groups was developed by Dr. Bart Haynes (Duke University School of Medicine). This monoclonal antibody is found to react with all cells infected with HTLV and producing products of this virus. Human umbilical cord lymphocytes infected with the virus by coculture also express altered HLA alloantigenic determinants. These alterations mirror those seen with tumor cell lines established from patients. Thus, the appearance of neoantigens suggests an association of HTLV provirus replication and HLA alloantigenic expression. Cell surface markers are examined on cell lines established from patients and cell lines established by coculturing with the human T-cell lymphoma virus. These cells are predominantly OKT4+ (helper phenotype). These cells also express increased HLA-DR and an antigen detected by the Tac antibody to the receptor for T-cell growth factor. A cytotoxic T-cell line, established from a patient with the HTLV-associated disease, kills autologous cells but is not cytotoxic for other cell lines established from patients with HTLV. The cytotoxicity appears to be genetically restricted by HLA determinants A1 and DR3. These investigations are providing new insights into the host factors that influence the pathogenesis of this disease.

In cultured cells from patients with classic adult T-cell leukemia, two lines were established that had B-cell surface markers, produced immunoglobulins and were infected with HTLV. Supernatants from these cell lines are found to

contain interferon alpha, both acid stable and acid labile. Further examination for soluble lymphokines produced by these cultured cells demonstrate production of B-cell growth factor. These observations suggest that B-cells may be infected in vivo with the T-cell tropic retrovirus. Furthermore, infection of B-cells with viruses can induce production of interferon alpha, a substance that is found in high levels in patients with certain autoimmune diseases and acquired immune deficiency syndrome (AIDS). Lymphokine production induced by virus infection may play a significant role in expansion of a transformed cell population to an apparent malignant state.

The human T-cell lymphoma virus HTLV-I has been found to be associated with patients with certain adult T-cell malignancies. Studies are underway to understand the mechanism of malignant transformation and immunologic response in individuals infected with this retrovirus. A novel HLA-class I epitope is expressed when cells are infected with the HTLV-I, but not with the HTLV-II, suggesting that the differences in the ENV region of the provirus are responsible for the expression of this epitope. This portion of the retrovirus encodes for the large envelope protein. HTLV-I virus has been found in B-cell lines that grew spontaneously from peripheral lymphocytes from patients with adult T-cell leukemia. These B-cell lines were infected with HTLV retrovirus as shown by Southern blot analysis using specific HTLV-III probes. Both cell lines also contained the Epstein-Barr virus. Supernatants from these cell lines were studied for production of lymphokines. Acid stable alpha interferon was found to be produced by one of the B-cell cultures and acid labile alpha interferon by the other B-cell culture. In addition, these B-cell lines produced B-cell growth factor. T-cell clones with specific functional activity were infected with the HTLV-I retrovirus. These infected T cells lost their ability to discriminate the activities possessed prior to infection. This loss of discrimination included the lack of recognition of specific antigen-presenting cells with a specific HLA-DR phenotype. The functions that were lost were specific cytotoxic function against histocompatibility determinants as well as response to KLH both in H3 thymidine incorporated and in helper T-cell response (i.e., the production of specific antibodies). The increased frequency of antibody to HTLV-I has been found in patients with chronic lymphocytic leukemia in the Carribean and in Japan, an area endemic for the HTLV retrovirus. B-cell chronic lymphocytic leukemia cells from a patient with HTLV-I antibodies were fused with the human lymphoblastoid cell line and the immunoglobulin produced by CLL cells expressed in the culture supernatants. These two such patients were studied. The immunoglobulin captured from one patient had specific antibody activity to the HTLV-I retroviral gag proteins 024 and cross-reacted with the gag proteins from HTLV-II and HTLV-III. Immunoglobulin captured from the second patient reacted specifically with HTLV-I-infected cell lines and not with the HTLV-II- and -III-infected cell lines. Low levels of activity were detected with this captured immunoglobulin against HTLV-I retrovirus. The differences in content of the large envelope protein on cells vs. the viral isolates suggest that this is the structure to which this immunoglobulin reacts. A shuttle vector was constructed which contained the ENV and 3' LTR of the HTLV-I retrovirus. This vector was used to transfect murine fibroblast lines and also contained a transforming virus. The transformed cells were selected and used to immunize mice from which monoclonal antibodies were developed. These monoclonal antibodies detect the small envelope protein p21 as well as p42 molecule. This latter molecule is thought to represent the product of the pX region of the retrovirus which is thought to have transacting capabilities acting on the 5' end of the LTR causing viral expression and in addition may act on other genes which appear to be preferentially expressed with HTLV-I

Immunobiology of AIDS and AIDS-Related Diseases

A large number of studies were performed examining the ratio of OKT-4-positive (helper) OKT-8-positive (suppressor) cells in homosexual populations from the Washington, DC and the New York areas. The total number of cells from these workers and the ratio of T-4 to T-8 (helper/suppressor ratios) were examined to attempt to correlate possible environmental or other risk factors. It was found that the Washington, DC male homosexual population had decreased numbers of OKT-4 cells in individuals who had homosexual contact with individuals in the New York, Los Angeles or San Francisco areas. All three of these areas are highly endemic for the acquired immunodeficiency syndrome. Two other subgroups could be identified--those being the intermediate-risk group and a low-risk group. The intermediate risk group are those who had homosexual contact with the individuals in the Washington, DC high-risk group. The low-risk group did not have homosexual contact with either of the two groups. There was a positive correlation of the low numbers of OKT-4+ cells among the individuals who had homosexual contact either directly or indirectly with individuals in the endemic areas. The sexual practices of these individuals were also related to the decrease in the OKT-4 population or helper/suppressor ratios. It was found that there was a positive correlation with increased numbers of homosexual partners as well as receptive anal intercourse. The sera from these individuals was studied for antibody against HTLV-III. Those individuals who had suppressed or decreased OKT-4-positive cells in their peripheral blood lymphocyte populations were generally positive for the HTLV-III retrovirus, showing a correlation of this retrovirus with the presence of one of the risk factors for this disease, i.e., the depression of the OKT-4-positive cells. Individuals who are at risk for the disease (male homosexuals) and lymphocytes from a heterosexual male population were used to study possible models for the depletion of the OKT-4 positive lymphocytes. Lymphocytes were placed in culture with a pan T-cell stimulator (PHA) and acid stabile and acid labile alpha interferon. Only those cultures of lymphocytes from AIDS patients and the male homosexual population with antibodies to HTLV-III showed depletion of the OKT-4 lymphocytes in vitro. These results suggest a model of antigen stimulation, together with the presence of the HTLV-III and alpha interaction, is neccessary for the depletion of the OKT-4+ cells. The depletion of OKT-4 lymphocytes suggested that the cell surface molecule bearing this epitope was a receptor for the HTLV-III retrovirus. Studies were performed to examine this possibility. Short-term incubation of the isolated banded retrovirus with the H9 cells demonstrated a rapid disappearance of epitopes on the OKT-4 molecule detected by antisera, OKT-4D, OKT-4F and OKT-4A. The epitope detected by the OKT-4 antisera disappeared after two to three days of exposure to the retrovirus. In all cells in which the retrovirus can be replicated, the OKT-4 molecule is absent. These results suggest that the specific receptor site for the HTLV-III retrovirus is on the distal portion of the OKT-4+ bearing molecule, that the molecule is internalized with the virus and that there is subsequent down-regulation of the expression of this molecule.

HLA Structure Function in Disease Association

HLA typing is performed on lymphocytes from patients with a common disease or families wherein one or more of the individuals shared a common disease type. One hundred and thirty individuals with mycosis tumorae were typed for HLA antigens. The HLA B35 antigen, as well as the DR5 antigen, was found to be increased in individuals with this disease compared to the normal population. HLA typing of seven multiplex families where family members had either melanoma

or the premalignant mole syndrome showed no linkage with the major histocompatibility complex. These data contradict early reports suggesting an MHC association or linkage with this disease. Families with adult T-cell leukemia and HTLV-I infection (not in leukemic patients) were typed for HLA antigens. A low degree of association (lode score, 1.5) was found with certain haplotypes in the MHC. In the family studies, three of twelve haplotypes had the HLA-1 antigen. None of the individuals inheriting this haplotype developed antibody to the HTLV-I retrovirus or had the disease. All indiviuals who were infected with the retrovirus were positive for the 4D12 monoclonal antibody. This monoclonal antibody detects an epitope on HTLV-I-infected cells that appears to be encoded for by the large envelope protein of the retrovirus. This epitope is also found on normal cells bearing the HLA-A19 and B35 cross-reactive group of allo antigenic determinants. We previously observed that HLA-1 is a genetic restricting determinant of cytotoxic T-lymphocytes to HTLV-I-infected cells. The family studies demonstrate the possibility of genetic control of infection of the HTLV-I retrovirus. HLA typing has been performed on 60 homosexual males and 45 individuals with AIDS. There is increased frequency of the HLA DR-4 antigens in the homosexual male population as compared to normals or the AIDS population. In contrast, HLA DR-5 is increased in frequency compared to the homosexual males without the disease and the normal population. The latter is particularly true in patients with Kaposi's sarcoma.

Descriptions of these and other findings are given in more detail in the individual project reports that are on file in the Office of the Director, Division of Cancer Etiology.

OTHER ACTIVITIES

The Laboratory has been responsible for training intramural and extramural investigators in the techniques for (1) culturing human epithelial tissues and cells and (2) enzyme immunoassays and fluorescent assays to measure carcinogen-DNA adducts. Members of the staff have also organized and/or served on the program committees of both national and international scientific meetings. These meetings include (a) Biochemical and Molecular Epidemiology of Cancer, (b) IV World Congress on Lung Cancer, (c) Toxicity, Tumor Promotion and Carcinogenesis, and (d) New aspects of Tobacco Carcinogenesis. During this fiscal year, members of the staff have also served as reviewers, on editorial boards of several journals and on intramural and extramural committees (e.g., NIH Handicapped Employees Advisory Committee, DCE Senior Promotion Review Committee, Environmental Pathology Committee of the International Academy of Pathology, and the International Advisory Board of the Danish Cancer Society).

UNIVERSITY OF MARYLAND (N01-CP-51000 replaces N01-CP-15738)

Title: Collection and Evaluation of Human Tissues and Cells from Patients with an Epidemiological Profile

Current Annual Level: $468,000

Man Years: 9.17

Objectives: To provide a resource to the NCI for the procurement, transport, and characterization of normal, preneoplastic, and neoplastic human bronchus, pancreatic duct, colon, and liver from patients with an epidemiological profile.

Major Contributions:

1. Collection of Tissues

Tissue specimens were collected from a total of 104 cases, including specimens from surgery patients (26) with and without cancer and at time of autopsy (78); autopsy specimens are collected from patients undergoing either immediate autopsy (10) (i.e., within 30 minutes after death) or routine autopsy (68) (i.e., between 2 and 12 hours after death). All tissues received at the NIH are usually residuals of materials taken for regular diganostic and corrective purposes and not for research per se.

A. Surgical Specimens

A total of 49 surgeries resulted in tissue donations.

Bronchus: Tumor tissues from 26 cases of lung carcinoma were collected. Bronchial specimens uninvolved with tumor were provided from all of these cases and transported to the NIH. Twelve specimens of lung tumor were received. The tumors were defined and classified as described below.

Colon: Tumor tissues from 23 cases of colon carcinoma were collected. Colonic tissue uninvolved with tumor from 22 of these cases was transported to the NIH. All of the tumors were defined and classified as described below.

B. Autopsy Specimens

Immediate Autopsy: There were 10 immediate autopsies. The specimens collected are shown below.

Organ	Number of Specimens
Colon	18
Bronchus with lung attached	10
Pancreatic duct	7
Liver	10

Tissues required for pathological examination and assessment of viability were retained by the contractor, but the major portions of the specimens were received at the NIH. Liver samples were quick-frozen in liquid nitrogen and stored at -70°C in the contractor's facility; other samples were shipped in ice-chilled L-15 medium.

Routine Autopsy: The numbers of specimens collected from 131 routine autopsies were as listed below:

Organ	Number of Specimens
Bronchus	68
Liver	63

1. Viability Evaluation

Bronchus: Small pieces of nontumorous bronchial epithelium from lung cancer patients (surgery) and from noncancer patients (immediate autopsy) were grown in explant cultures. Eighty percent of the cases were viable.

Pancreas: Pancreatic ducts were collected from 10 immediate autopsy cases. Pancreatic ductal tissue was viable in serum free media of either the CMRL 1066 or Parsa based recipe. CMRL 1066 was better with 7 factors (insulin, hydrocortisone, transferrin, phosphaethanolamine and ethanolamine, bovine pituitary extract, epidermal growth factor) than either Parsa media or CMRL 1066 without growth factors.

Parsa media (Cancer 47: 1543, 1981) has been routinely less effective in explant organ culture than CMRL 1066. Controls to assess viability in CMRL 1066 with and without serum and growth-promoting factors (insulin, transferrin, EGF, etc.) were included. CRML 1066 with 5% heat-inactivated fetal bovine serum was the most effective.

Pancreatic tissue from routine autopsies are uniformly nonviable. Ischemic changes, areas of fat necrosis, and nonviable explant cells were observed in all cases.

Liver: Nine liver specimens from immediate autopsies and 63 from routine autopsies were frozen and stored. Liver slices and cells were stored by the contractor and are available upon request for shipment to the NIH.

In the 12-month period, 7 livers from immediate autopsy were used for successful hepatocyte isolation. Post-mortem changes were observed in several, but viable hepatocytes were isolated from these tissues. Viability was determined by the ability to isolate viable cells from a lobe of the liver by a 2-stage perfusion method (Hsu et al., 1985). Perfusion is initiated via a branch of the portal vein and Ca++ free Hank's salt solution is infused for 15-20 minutes. Collagenase (185 u/ml) is then infused for 20-25 minutes and recirculated if necessary. Cells are obtained by mincing perfused sections and spinning in a flask for 10 minutes. Cell viability is determined by trypan blue exclusion (positive > 70%). The study of detoxification enzymes and ultrastructure of cultured hepatocytes is continuing.

3. Epidemiological Profile Construction and Storage

Abstracting medical records, compiling donor histories, and computerizing these data continue as essential requirements of this project. In this period, 73 medical records were abstracted for surgery patients (60 for thoracic and 13 for colonic); donor histories were compiled for 43 patients (22 bronchus and 21 colon) via interviews using the standard questionnaire (developed by LHC and the contractor); and in data processing, a total of 83 (19 colonic and 64 thoracic) records (medical and epidemiological) have been coded for computer storage and analysis.

The total number of cases with these data collected from the beginning of the contract from the 6 participating hospitals are listed below:

	Univ. Hosp.	LRVA	Un. Mem.	St. Agnes
Bronchus	274 (91)	116 (28)	23	47 (25)
Colon	275 (105)	79 (14)	0	0

	W. Va.	Sinai	Med. Exam.	Total
Bronchus	0	1	138	599 (283)
Colon	36		0	390 (155)
Total				989

( ) = 4 year totals for the expired contract.

To date, the activities completed in the effort to provide epidemiological profiles for donors of tissues delivered in this period are as follows:

	Med. Rec.	Interviewed	Coded	Interview Refused By: Patient	Doctor	Hospital	Med. Exam.
Bronchus	572 (273)	362 (142)	558 (295)	36 (14)	5 (1)	23	109
Colon	346 (145)	294 (144)	340 (176)	21 (6)	5 (2)	0	0
Total	918 (418)	656 (286)	898 (471)	57 (20)	10 (3)	23	109

4. Definition and Classification of Nonneoplastic and Neoplastic Tissue

Epidemiologic data are provided to allow determination of the relationships between tumor type and selected risk factors and the amount of benzo[a]pyrene (BP) or aflatoxin B_1 (AFB), respectively, (e.g., in lung cancer and hepatoma), bound to DNA by the same patient's noncancerous epithelium.

Bronchus: The contractor provided the following characterizations:

Morphological and histochemical characterization of human primary lung carcinomas are routine. Characterization of tissues by immunocytochemistry has continued using the peroxidase-antiperoxidase method to demonstrate the presence or absence of various antigens. Tumor and normal tissues, abnormal and preneoplastic tissues are examined for beta human chorionic gonadotropin (HCG), calcitonin, adrenocorticotropic hormone (ACTH), serotonin, alphafetoprotein (AFP), keratin, somatostatin, neuron specific enolase (NSE), calmodulin, and tubulin.

Normal and abnormal (but nonneoplastic) adult bronchi contain only mucosubstances, keratin, calmodulin, and tubulin. Keratin is seen only in aldehyde-fixed bronchi if the epithelium is neoplastic; in the basal layer of ethanol-fixed bronchi, keratin is seen in normal bronchial epithelium (including bronchial glands) and occasionally in columnar cells that reach the lumen. Calmodulin apparently increases levels at the cell borders.

Lung tumors have greater heterogeneity than tumors in the bronchial epithelium. Each marker is found at least occasionally in tumors, HCG is found in 80% of non-small cell tumors, and keratin is found in 75% of such tumors. Somatostatin is seen in keratinizing areas and more diffusely in a smaller proportion of adenocarcinomas. NSE and serotonin are seen in endocrine tumors only, and only in tumors with dense-cored granules, including small cell carcinomas, carcinoids and atypical endocrine carcinomas. Appearance of other markers (observed less commonly--ACTH: 40%, somatostatin: 40%, calcitonin: 20%, AFP: 2%), shows less predilection for types of lung tumors, and HCG seems to follow glycogen distribution.

Indirect immunofluorescent detection of tubulins was performed on cellular outgrowths of bronchial explants involved and uninvolved with tumor. Generally uniform, the nonmalignant cells had mostly straight microtubules originating from assembly sites near the nucleus, while the variable tumor cells had irregular microtubular patterns in a mesh-like arrangement.

Colon: All tissues were characterized as described below:

1. Morphological (light microscopy [LM], transmission electron microscopy [TEM] scanning electron microscopy [SEM]) and histochemical examinations of normal, premalignant, and malignant human epithelium continue. The comprehensive description of the morphology of normal human colon is still incomplete, and some of the previously reported morphological markers of premalignancy may ultimately be declared normal aspects of different tissue segments. This possibility is suggested in electron microscopy (EM) data showing 3 colonic cell types (undifferentiated, endocrine, and mucous). In ascending segments, apical vesicles are EM dense on electron microscopic examination, but in the rectum, they are EM lucent. By LM, using histochemical stains, there are differences seen in ascending, transverse, and descending cells with mixed magenta and blue-purple staining; in the rectum almost all cells stained blue, indicating highly acid mucus. HID-AB staining showed a large proportion of cells in all 4 regions staining brown-black, indicating high amounts of sulphomucin. A study of different fixatives (4F + 1G, 10% neutral formalin, 100% ethanol and acid 95% ethanol) for their individual effect on CEA staining showed that staining increases progressively from 4F = 1G to acid alcohol, which preserves the antigen to the greatest extent.

2. Human ascending, transverse, and rectal colonic epithelium from immediate autopsies are being maintained routinely in explant culture and provided to the NCI for xenotransplantation.

Pancreas: All tissues received were characterized as described below. Pancreatic tisses from immediate autopsy were examined by morphological techniques, histochemistry, immunohistochemistry, and freeze fracture. Pancreatic ducts were maintained using the contractor's organ explant and cell culture techniques, histocompatibility, immunohistochemistry, and freeze fracture. Routine autospy

and surgically derived tissues were examined to elucidate cellular alterations in pancreatic cancer. Previously, of 40 cases, primary and metastatic tumor (18 autopsy cases and 22 surgical cases), studied in routine LM, over 90% looked like duct cell adenocarcinomas.

Liver: Samples were collected and portions stored at -70°C. The methods for culturing liver tissue and cells are still under study as indicated below.

Comparison of methods for the primary culture of human hepatocytes and rat hepatocytes are continuing using different media and substrates, including human liver biomatrix. EM of zero-time samples is used to assess the viability of liver tissue at the time of perfusion. Comparisons are being made for optimal cell isolation between perfusion of intact lobes and wedge-shaped sections of lobes. Pieces of liver are also quick-frozen in liquid nitrogen for subsequent use in metabolic studies at the NCI. Preliminary results indicate that primary cultures of human liver cells can provide a mechanism for studying chemical metabolism and mutagenesis.

Proposed Course: In the coming year, emphasis will be placed on organizing to improve this resource toward increased contribution to the NCI research program.

UNIVERSITY OF MARYLAND (N01-CP-31008)

Title: Resource for Human Esophageal Tissues and Cells from Donors with Epidemiological Profiles

Current Annual Level: $83,615

Man Years: 1.27

Objectives: To provide tissue specimens and cells of human esophagus from epidemiologically defined donors to the Laboratory of Human Carcinogenesis for carcinogenesis studies; to provide fresh, well-characterized, and viable esophageal tissue for primary organ culture at the NIH to create, characterize, and store monolayer cultures from esophageal tissues for delivery on request to the NIH.

Major Contributions: Forty-four specimens were collected and characterized by the contractor. An epidemiologcal profile of the donors has been provided whenever possible. Tissues from the Medical Examiner (ME) source possess chance viability in organ cultures, which decrease rapidly with time after death. Esophagi collected more than 8 hours post-mortem are unlikely to survive in vitro.

Monolayer cultures are developed according to the methods of Dr. Susan Banks-Schlegel (Exp. Cell Res. 146:271-280, 1983). Explants from 66 "normal" uninvolved and 67 malignant human esophageal mucosa were cultured in this period. The cell banking facility is an integral part of this contract. Primary suspension cultures of cells from normal and malignant esophageal mucosa are frozen and thawed as cell stocks in viable condition. Cells were obtained from both normal human esophagi (immediate autopsies) and esophageal cancers. Currently, there are approximately 1,249 vials of frozen stock, including 1,092 epithelial cell suspensions (237 normal, 855 malignant), 76 3T3 feeder cells, and 81 sarcoma-180 mouse tumor cells for the production of tumor-conditioned medium, both required for esophageal cell culture.

Morphological, cytochemical, and immunocytochemical characteristics were determined for each tissue collected and will be delivered to the NCI or elsewhere on request. Assays are also being conducted to determine specific biochemical markers occurring in normal, premalignant, and malignant human esophageal epithelium.

Proposed Course: Continuation of tissue collection; careful, thorough in vitro testing of the quality of the frozen cells for in vitro carcinogenesis experiments.

GEORGETOWN UNIVERSITY (N01-CP-31007)

Title: Collection and Evaluation of Human Tissues and Cells from Donor with an Epidemiological Profile

Current Annual Level: $64,952

Man Years: 0.96

Objectives: To provide the NCI with (1) a source of human lung and bronchial tissues taken at surgery, (2) pleural fluid from patients with benign and malignant lung disease, (3) human bronchoalveolar cells from bronchial lavage of normal smokers and nonsmokers, and (4) completed epidemiological questionnaires for medical and environmental histories.

Major Contributions: In this period, the contractor provided 11 specimens (1 benign, 10 malignant) from resected bronchus and peripheral lung, 19 specimens of pleural fluid from mesothelial cells, 31 sets of lavage samples (bronchoalveolar cells) and corresponding blood (mononuclear cells) preparations from 12 smoking and 19 nonsmoking normal volunteers. Epidemiological (medical and environmental history) profiles were completed for all participating patients and normal volunteers. These records were filed in the contractor's facility for future use by the NCI.

Materials obtained from this contractor were used in ongoing studies in the In Vitro Carcinogenesis and Biochemical Epidemiology Sections of LHC. The human bronchial and peripheral lung tissues are used in ongoing studies of chemical carcinogenesis and human lung cancer. The mesothelial cell cultures from pleural fluids are used to examine in vitro effects of asbestos and other environmental agents that may be involved in the pathogenesis of malignant mesothelioma. The bronchoalveolar and peripheral blood mononuclear cells and the bronchoalveolar cells from smokers and nonsmokers were used in biochemical epidemiology studies to develop methods for monitoring human exposure to carcinogens and mutagens. The specimens are tested for carcinogen DNA adducts and their ability to repair DNA damage.

Proposed Course: To continue and improve provision of the specified tissues and cells to the NCI during the next contract period.

LITTON BIONETICS (N01-CP-15769)

Title: Resource for Xenotransplantation Studies of Carcinogenesis in Human Tissues in Athymic Nude Mice

Current Annual Level: $294,320

Man Years: 2.43

Objectives: To provide an immunodeficient animal model, athymic nude mouse, (1) for long-term survival of human tissue xenografts; (2) for a continuing resource of athymic nude mice for long-term xenotransplantation, proliferation, and tumorigenicity studies of normal, premalignant, and malignant human tissues; (3) to use human tissues to study the in vivo development of preneoplastic and neoplastic transformation induced in vitro and in vivo by carcinogens; and (4) to study the ability of selected agents and cellular manipulations to modify the effects of carcinogens on human tissues.

Major Contributions: Human bronchus, pancreatic duct, colon, breast, prostate, and esophagus are maintained for 16 months and beyond as xenografts, as evidenced by viable-appearing epithelium with normal histology and the incorporation of labeled precursors into epithelial cells of the grafts.

Epithelium-denuded rat tracheas successfully serve as anchorage for human bronchus cells, which attach to and layer the luminal surface of the tracheas. Esophageal xenografts are characterized by epithelial growth of cyst formation.

In the breeding stock, 472 Swiss litters contained 3,015 pups (6.9/litter), including 1,416 nu/nu pups. Eighty-nine percent of these newborns survived, giving a total of 1,310 or 2.9 surviving nudes/litter. During the year, the contractor maintains a monthly average colony population of 626 mice: 100 breeders, 174 newborns, 110 weanlings for new experiments, and 242 mice in experimental protocols. An average of 8 experimental animals died and 47 were killed monthly.

In this period, 34 new experiments were initiated requiring a total of approximately 396 mice. These included 10 new (36 ongoing) experiments to study the growth rate and morphology of xenotransplanted HUT 294 cells, a bronchial epithelial cell line, implanted with and without a variety of X-irradiated feeder cells, irradiation of the host and treatment of the host with anti-interferon, antiserum or the implant with dimethylnitrosamine, cigarette smoke condensate, nickel (Ni), chromium, or arsenic; 20 new (30 ongoing) experiments to study (a) the morphology of xenotransplanted normal human esophageal (HE) tissue after treatment with N-methyl-N-nitro-N-nitrosoguanidine (MNNG), dimethylbenzanthracene, or dimethylsulfoxide; (b) growth of HE cells in antilymphocyte serum (ALS)-treated or X-irradiated mice; and (c) growth of HE carcinoma cells in normal nude mice; seven experiments to investigate xenographic characteristics of human mesothelial, pancreatic, and hepatic cells in nude mice. Other ongoing studies include experiments to study xenographic growth and morphology of human pancreas and bladder tissues with and without MNNG treatment of exposure to SV40 + Ni_3S^2 before xenotransplantation into ALS-treated nude mice. One ongoing experiment is designed to study xenographic morphology and growth of human prostate.

The contractor maintained a 6 month average of 83 ongoing experiments requiring 927 mice, 604 of which survived the 6 month period.

Malignant transformation from tissue treated chemically in vitro continues to elude observation in xenografts. Tissue explants treated in vitro with carcinogens respond with epithelial abnormalities which, when xenografted infrequently maintain transformation in the nude mouse for sufficiencly extended periods. Squamous metaplasia occurs in grafts given carcinogens in vivo but have not become malignant. Emphasis continues to be placed on increased immunosuppression as a means of effecting the change.

However, in this period, the successful xenotransplantation of oncogene transfected human bronchial epithelial (HBE) cell lines continues to produce tumors. One hundred percent of the nu/nu mice given subcutaneous injections of HBE cells transformed in vitro by transfection with the Harvey ras gene developed progressively growing nodules. Even those mice give inoculums of very early passages of transformed cells without soft agar selection have developed tumors during extended observations.

Proposed Course: Continuation of the following resource activities: (1) long-term testing of the effects of carcinogens and treatment regimens in vivo on human tissue xenografts in the athymic nude mouse; (2) examining for tumorigenic potential of human cell exposed to chemical and physical carcinogens in vitro; (3) xenotransplantation of transformed and nontransformed cells combined with other cells (species and type) with and without treatment with carcinogens, enzymes, or ionizing irradiation (cesium source); and (4) increased emphasis on further immunosuppression of the nude mouse to enhance its xenotransplantation capabilities. In this area, antimouse interferon antibodies, coinjection of human fibroblasts, antilymphocyte serum, and various monoclonal antibodies are being explored for this purpose.

NATIONAL NAVAL MEDICAL CENTER (Y01-CP-30257); WALTER REED ARMY MEDICAL CENTER (Y01-CP-30504)

Title: Procurement of Human Tissues from Donors with an Epidemiological Profile

Current Annual Level: $16,951

Man Years: 1.0

Objectives: (1) To provide specimens of nontumorous bronchial and colonic epithelium (obtained at time of surgery for cancer or for benign lesions), with epidemiological profiles of medical and environmental histories for each donor, to the NIH for the study of carcinogen activation and deactivation and (2) to determine the ability of human tissue to metabolize carcinogens to mutagens.

Major Contributions: Because of a Navy staffing problem previously reported and now known to have concluded with the dismissal of the PI for the agreement, the National Naval Medical Center (NNMC) has essentially terminated the administrative management and control responsibility they held under the original agreement. LHC is continuing its participation in the agreement with the transfer of administrative responsibilities to Walter Reed where the tissue collection technicians will be hired and supervised for tissue collection from both surgeries.

Proposed Course: A current agreement to transfer hiring and supervision of the tissue collection technician to Walter Reed (Y01-CP-30504) cardiothoracic surgery department is now in place. It is expected that, when the technician is hired, Walter Reed Army Hospital will increase the tissue yields and perhaps stabilize the technical aspects with more experienced management. In addition, specimens obtained from Walter Reed will increase the total volume without added cost.

VETERANS ADMINISTRATION HOSPITAL (Y01-CP-30255)

Title: Resource for Procurement of Human Tissues from Donors with an Epidemiological Profile

Current Annual Level: $59,535

Man Years: 1.6

Objectives: This interagency agreement provides (1) specimens of normal, premalignant, and malignant human lung and colon tissues (taken at the time of surgery) for the study of human epithelial responses to carcinogens in cell and organ cultures and as xenotransplants in immunodeficient mice; (2) morphologic and pathologic characterization by light and electron microscopy and histochemistry of normal, premalignant, and malignant epithelium for each tissue; and (3) an epidemiological profile (including preoperative medical and environmental histories) for each donor.

Major Contributions: From lung and colon surgeries, the contractor delivered a total of 136 specimens: 50 lung (25 normal, 25 malignant); 36 colon (20 normal, 16 malignant); 15 bronchus and 28 pleura. All tissues received were characterized by light and electron microscopy. Of the 81 patients who variously underwent pneumonectomy, lobectomy, bilobectomy, and local excision, 18 (22%) had squamous cell carcinoma, 13 (16%) had adenocarcinoma of the lung, and 17 (20%) had adenocarcinoma of the colon. Tissues delivered to the NCI came from cases with a sufficient excess after clinical diganosis and characterization purposes were satisfied. Cooperating donors have epidemiological profiles completed and filed in the contractor's facility.

Thus, the contractor continues to obtain acceptable numbers and kinds of tissues from the surgical procedures involved, which in this period were as follows:

Right pneumonectomy	3
Left pneumonectomy	5
Right upper lobectomy	13
Right middle lobectomy	2
Right upper lobectomy	6
Left upper lobectomy	7
Left lower Med	1
Mediastinal resection	3
Local excision	9
Colectomy	23
Gastro-esophagectomy	1
Total	73

By light microscopy, it was ascertained that 20 of the 73 patients did not harbor a malignancy, but rather had a number of other conditions ranging from normal epithelium to granuloma. The pulmonary diagnoses were identified as follows:

Squamous cell carcinoma	18
Adenocarcinoma (lung)	13
Adenocarcinoma (colon)	17
Undifferentiated small cell	1
Undifferentiated large cell	1
Thymoma	2
Hematoma	1
Granuloma (pulmonary)	10
Emphysema	1
Lipoma	1
Colitis	1
Diverticulitis	2
Pneumonia	1
Normal epithelium	2
Total	71

Proposed Course: Based upon mutual experiences to date, the contractor's activity should include the following: (1) continue obtainment and morphological examination of viable normal and abnormal human tissues from patients with an epidemiological profile and (2) increase obtainments to include more samples of esophageal, gastric, and colonic mucosa.

UNIFORMED SERVICES UNIVERSITY OF THE HEALTH SCIENCES (YO1-CP-00502)

Title: Hybridoma Resource

Current Annual Level: $127,755 (90,000)

Man Years: 0.75

Objectives: To prepare and screen monoclonal antibodies to cell surface antigens and alkylated DNA for use in studying carcinogen and cocarcinogen actions in bronchial epithelium, including (1) identification of normal preneoplastic and neoplastic bronchial epithelial cells; (2) characterizations of transforming growth factors secreted by human cancer cells; and (3) measurement of DNA damage in normal and neoplastic bronchial epithelial cells caused by carcinogens and antitumor drugs.

Major Contributions: Mice have been injected with tobacco smoke condensate (TSC) adducted to DNA mixed with protamine, the latter serving as the adjunct. Spleen cells from mice producing antibody were fused with the HAT-sensitive NS-1 cells, cloned, and expanded, and the supernatants were screened for antibody. Further characteristics of specificity are being examined. In other studies, O^6-ethylguanosine and N^7-ethylguanosine were complexed specifically with protamine and used to immunize mice. Monoclonal antibodies resulting from this immunization were produced by the techniques described above. These antibodies react with ethylated-DNA. Specificity determinations are under way. Sera from individuals exposed to high levels of benzo[a]pyrene were assayed for antibodies to this compound adducted to DNA. Some of these individuals as well as others with lower levels of exposure were found to react with this compound.

Proposed Course: To develop monoclonal antibodies to a number of DNA adducts formed by environmental carcinogens, including those found in tobacco smoke; to produce monoclonal antibodies to the alkylated DNA adducts and surface antigens from chemically treated cells; and to analyze cell surface antigens for possible correlations with chemically induced DNA modifications.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP04513-10 LHC

PERIOD COVERED

October 1, 1984 to Sepetember 30, 1985

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Metabolism of Chemical Carcinogens by Cultured Human Tissues and Cells

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	Curtis C. Harris	Chief	LHC	NCI
Others:	Kirsi Vahakangas	Visiting Fellow	LHC	NCI
	Simon Plummer	Visiting Fellow	LHC	NCI

COOPERATING UNITS (if any) Univ. of MD School of Medicine, Baltimore, MD (B.F. Trump); Institute for Cancer Research, NY, NY (A. Jeffrey); Cancer Institute, PRC (Sun Tsung-tang and Hsia Chu-chieh); Dept. of Surgery, Univ. of Nairobi, Nairobi, Kenya (J. Wakhisi); Fiberger Institute, Copenhagen, Denmark (H. Autrup)

LAB/BRANCH

Laboratory of Human Carcinogenesis

SECTION

Carcinogen Macromolecular Interaction Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.0	1.0	

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Human bronchus, colon, duodenum, esophagus, and pancreatic duct cultured either as explants or epithelial cells in chemically defined media provide an excellent in vitro system to study the metabolism of chemical carcinogens, including those found in tobacco smoke and the environment. Several classes of chemical carcinogens, polynuclear aromatic hydrocarbons, N-nitrosamines, hydrazines, aromatic amines, and mycotoxins, can be metabolically activated by human tissues. Fetal human liver, stomach, and esophagus cultured as explants metabolized the same group of compounds. The metabolic pathways leading to the formation of DNA adducts in explants and epithelial cell cultures have been defined for benzo[a]pyrene (BP), 7,12-dimethylbenz[a]anthracene, aflatoxin B1 (AFB), and N,N-dimethylnitrosamine (DMN). The adducts between these carcinogens and DNA in human tissues are essentially the same as those found in experimental animals in which the chemicals are carcinogenic. Interindividual differences in carcinogen-DNA binding values vary 50- to 150-fold. Studies of the metabolism and DNA adduct formation of 1-nitropyrene and 6-nitrobenzo[a]pyrene has also shown similar findings in interindividual and interspecies comparisons. The role of AFB in liver carcinogenesis has been further studied. Fetal liver explants have also been shown to activate AFB, DMN and BP. Since human fetus may be transplacentially exposed to these carcinogens, this finding may be important in the etiology of liver cancer and the early age of onset in China. We found that when urine samples collected in Kenya were analyzed for the presence of 2,3-dihydro-2-(7'-guanyl)-3-hydroaflatoxin B1 (AFB-Gua I) by high pressure liquid chromatography, 11 of 128 samples had a detectable level of AFB-Gua I; its identity was confirmed by photon-counting fluorescence spectrophotometry. The positive samples were primarily from people living in low-lying areas and collected in the rainy season when AFB contamination of the food is highest.

PHS 6040 (Rev. 1/84)

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

Curtis C. Harris	Chief	LHC	NCI
Kirsi Vahakangas	Visiting Fellow	LHC	NCI
Simon Plummer	Visiting Fellow	LHC	NCI

Objectives:

To determine the metabolic pathways of chemical carcinogens in target tissues of experimental animals and humans. To measure interindividual and intertissue variations in the metabolism of carcinogens.

Methods Employed:

Explant cultures and epithelial cell cultures of human and animal tissues; isolation of cellular macromolecules; high pressure liquid chromatography; enzyme systems; and synchronous scanning fluorescence spectrophotometry.

Major Findings:

Cultured human bronchial mucosa can enzymatically activate procarcinogens (polynuclear aromatic hydrocarbons: 7,12-dimethylbenz[a]anthracene [DMBA], 3-methylcholanthrene [MCA], benzo[a]pyrene [BP], 6-nitrobenzo[a]pyrene 6-NO_2BP], 1-nitropyrene and dibenz[a,h]anthracene [DBA]; N-nitrosamines: N-nitrosodimethylamine [DMN], N-nitrosodiethylamine, N-nitrosopiperidine, N-nitrosopyrrolidine [NPy], and N,N'-dinitrosopiperazine; a substituted hydrazine, 1,2-dimethylhydrazine [1,2-DMH]; a mycotoxin, aflatoxin B_1 [AFB]; and an aromatic amine, 2- aminoacetylfluorene) into metabolites that bind to cellular macromolecules, including DNA.

The extrapolation of carcinogenesis data among animal species depends in part on qualitative differences between metabolic activation and deactivation of procarcinogens. As shown previously, the metabolism of BP has been extensively studied in explants of tracheobronchial tissues from experimental animals-hamsters, rats, mice, cows--and humans. The total metabolism as measured by both organic solvent-extractable and water-soluble metabolites of BP was substantial in the respiratory tract from humans and animal species susceptible to the carcinogenic action of BP. Furthermore, the results suggested that determination of both activation and deactivation pathways is important in assessing carcinogenic risk of a chemical. No qualitative difference in the profile of organosoluble metabolites (tetrols and diols being the major metabolites) was observed among the different species. The metabolism of BP-7,8-diol to BP tetrols was mediated not only by the mixed function oxidase system but also by the prostaglandin synthetase pathway. Addition of arachidonic acid to the culture medium enhanced the production of the BP-tetrols. The prostaglandin synthetase pathway did not activate BP itself. The binding values of BP to cellular DNA were quite similar

in all tissues, although slightly higher binding was observed in hamster trachea. Wide interindividual variation in the binding of BP to DNA was seen in tissues from outbred species. The major BP-DNA adducts in all animal species were formed by interaction of BP diol-epoxide with the 2-amino group of deoxyguanosine. Both stereoisomeric forms of (±)-7β ,8α)-dihydroxy-(9α,10α)-epoxy-7 8,9,10-tetra-hydrobenzo(a)pyrene (BPDE I) reacted with deoxyguanosine, the (7R)-form being the most reactive. No difference in the relative distribution of the various adducts was seen among the species, except in the rat (DC Wistar and Buffalo), where BPDE-deoxyadenosine adducts accounted for 20% of the total modification. In conclusion the metabolism of BP is qualitatively similar in tracheobronchial tissues from both humans and animal species in which BP has been experimentally shown to be carcinogenic. The major DMBA-DNA adduct was also formed between the "bay-region" diolepoxide and the 2-amino group of deoxyguanine.

The metabolism of BP was studied in both epithelial and fibroblast cells initiated from the same bronchus specimens. The total metabolism was three-fold higher in the epithelial than in the fibroblast cells. No qualitative differences in the metabolic profile of BP between the explant culture and the epithelial cell cultures were observed. Transformed human bronchial epithelial cells did not metabolize BP as efficiently as normal cells in vitro.

Nontumorous esophagus cultured in a chemically defined medium metabolized BP DMBA, AFB, DMN and N-nitrosodiethylamine (DEN) to species that reacted with DNA. No detectable amount of radioactivity was associated with DNA after incubation with NPy. The major carcinogen-DNA adducts were (1) trans addition of (+) BP diol epoxide I at the 10 position to the 2-amino group of guanine (2) with DMBA addition of DMBA-3 4-dihydroxy-1,2-epoxide to the 2-amino group of guanine; (3) with DMN 7-methylguanine and O6-methylguanine (O6-MeG/7-MeG = 0.3), and (4) with AFB, 2,3-dihydro-2-(N-guanyl)-3-hydroxy-aflatoxin B_1.

The mean level of binding of BP in human esophageal DNA was lower than that in bronchus from the same individual and showed a 100-fold interindividual variation. As N-nitrosamines are potential esophageal carcinogens in rats, a comparative study in humans and rats on the metabolism of this group of compounds was performed. Both acyclic and cyclic N-nitrosamines were metabolized by rat esophagus. The highest level of metabolite binding was seen with N-nitroso-benzylmethylamine (BMN), an organotrophic carcinogen for the rat esophagus. The binding level was about 100-fold higher than in adult human esophagus. The results indicate significant quantitative and perhaps qualitative differences between cultured rat and human esophagus in their ability to activate N-nitrosamines.

Metabolism of various carcinogens in cultured human colon and duodenum has been investigated. Nontumorous tissue was collected at the time of either "immediate autopsy" or surgery from patients with or without colonic cancer. After 24 hours in culture explants were exposed to radioactive-labeled carcinogen for another 24 hours, and the binding to cellular DNA was measured by radiometric methods. The following carcinogens were converted by human colon to species that bound

to DNA: BP, 6-NO-BP, 1-nitropyrene, DMBA, AFB, DMN, DMH, and 3 amino-1,4-dimethyl-5H-pyrido(4 3b)indole. The latter is a potent fecal mutagen formed by pyrolysis of tryptophan. The major carcinogen-DNA adducts were identified for BP, DMBA and AFB and were found to be identical to the adducts formed in human bronchus.

The role of prostaglandin H synthase (PHS) in the metabolism of 7,8-dihydroxy-7 8-dihydrobenzo(a)pyrene (BP-7,8-diol) has been examined in short-term explant cultures of hamster and human tracheobronchial tissues. Labeled BP-7 8-diol was incubated with the explants in the presence and absence of the PHS substrate arachidonic acid (20:4) and the PHS inhibitor indomethacin. The addition of 10 μM to 200 μM 20:4 to incubations of hamster trachea with 5 μM BP-7,8-diol caused significant increases in the formation of 7r,8t-dihydroxy-9t 10t-epoxy-7 8,9,10-tetrahydrobenzo(a)pyrene (anti-BPDE). These increases were not seen when 1 μM or 20 μM BP-7 8-diol was employed. The stimulation of anti-BPDE formation was observed after incubations of from 1 to 48 h. This stimulation was inhibited to the basal level by 20 μM indomethacin, supporting the role of PHS in the response. No effect of 20 4 was seen on the uptake of BP-7,8-diol by the tracheas or on the formation of water-soluble metabolites. Significant increases in covalent binding of BP-7,8-diol metabolites to DNA of the tracheal epithelium were also elicited by the addition of 20:4; however, these increases were not well correlated quantitatively with the increases in anti-BPDE formation. HPLC profiles of deoxynucleoside adducts from basal and 20:4-stimulated incubations ere qualitatively identical. Far greater variability of metabolism was seen in human bronchus explants, but 20:4-dependent increases in anti-BPDE formation could be demonstrated in those tissues as well. Inhibition of this stimulation by indomethacin was either absent or incomplete. This variation in the effect of indomethacin was explained by the examination of the products of 20:4 metabolism by the two tissues. Hamster trachea produced almost exclusively PHS metabolites, whereas human bronchus yielded predominantly products of lipoxygenases, enzymes insensitive to indomethacin. In conclusion, this study indicates that cooxygenation of chemical carcinogens can occur in hamster and human tracheobronchial tissues. The concentration dependence observed with BP-7,8-diol, however, suggests that this pathway is of minor importance in the activation of BP in these tissues.

The mean level of binding of BP was higher in duodenum than in colon. A wide interindividual variation was observed. A positive correlation in the binding level of BP between bronchus and colon and duodenum from the same individual was seen.

Explant cultures of human fetal liver, stomach and esophagus extensively metabolized chemical carcinogens into DNA binding species. The metabolism of N-nitrosamines--DEN, NPy and BMN--was significantly higher in stomach than in the other tissue from the same fetus. Fetal esophagus did metabolize NPy into a DNA-binding metabolite in contrast to adult esophagus. The major BP-DNA and AFB-DNA adducts in fetal livers were similar to the adducts observed in other adult human tissues. The high pressure liquid chromotographic profile of organosoluble BP-metabolite was more complex than with adult tissues.

In order to assess the role of AFB in human liver carcinogenesis, we collected urine samples in Kenya for analysis of AFB-Gua I, a "DNA-repair product." It has previously been shown that food samples collected are known to be contaminated with AFB, and a positive correlation exists between the dietary intake of AFB and the incidence of liver cancer. The urine samples collected at the Outpatient Clinic of Murang'a District Hospital was concentrated on C_{18}Sep-Pak columns and AFB-Gua I was isolated by high pressure liquid chromatography in two different systems. Eleven of 128 samples had a detectable level of a compound whose synchronous fluorescence spectrum was identical to chemically synthesized AFB-Gua I. The spectrum did not show any batochromic shift when pH was made alkaline. These results are an indication of interactions between the ultimate carcinogenic form of AFB and cellular nucleic acids in vivo and further support the hypothesis that AFB may play an important role in the etiology of human liver cancer.

Significance to Biomedical Research and the Program of the Institute:

Because most environmental carcinogens require metabolic activation to exert their carcinogenic effect, the study of their metabolic pathways and the reaction of the ultimate carcinogen with cellular macromolecules in potential human target tissues is important. Extension of these studies to more complex, potentially carcinogenic mixtures, such as tobacco smoke condensate, is important. The development of controlled culture conditions for human tissues provides a model system for these studies in intact human tissues. The use of explant culture also provides a link between studies in experimental animals and the human situation as the metabolism of the carcinogens can be studied at the same level of biological organization in both species; this information is essential for extrapolation of carcinogenesis data between species. Furthermore, human tissues obtained by immediate autopsy also will allow a comparative study in various organs from the same individual. From this study, we hope to be able to identify an easily accessible cell type that can be used for the identification of idividuals at high risk of developing chemically induced cancers.

Proposed Course:

Our attention will continue to focus on chemicals found in tobacco smoke and environmental agents, such as T_2 toxin and fecapentaene. To compare the metabolism of chemical carcinogens in target tissues and possible "detector" cells, i.e., monocytes and macrophages, a study on the biological effect in relationship to level and type of carcinogen-DNA interaction will be continued.

Publications

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05192-05 LHC

PERIOD COVERED

October 1, 1984 to Sepetember 30, 1985

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Repair of Carcinogen-Induced Damage in Human Cells

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	Curtis C. Harris	Chief	LHC	NCI
Others.	Hans Krokan	Guest Researcher	LHC	NCI

COOPERATING UNITS (if any)

Department of Physiology, Hershey Medical Center, Hershey, PA (A.E. Pegg), Department of Pathology, University of Maryland School of Medicine, Baltimore, MD (B.F. Trump); Karolinska Institute, Stockholm, Sweden (R.C. Grafstrom)

LAB/BRANCH

Laboratory of Human Carcinogenesis

SECTION

Carcinogen Macromolecular Interaction Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.0	1.0	

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Normal adult human tissues and cultured bronchial epithelial cells and fibroblasts exhibit O6-alkylguanine-DNA alkyltransferase activity in vitro by catalyzing the repair of the promutagenic alkylation lesion O6-methylguanine from DNA. Alkyltransferase activity varies in the different human tissues tested in the decreasing order of liver > colon > esophagus > peripheral lung > brain. Various human tissues exhibit 2- to 10-fold higher alkyltransferase activity than corresponding rat tissues. The present results show that different human tissues and cells have a several-fold higher capacity to repair O6-methylguanine in DNA than rat tissues and that the repair process occurs via a mechanism similar to that previously shown in other mammalian cells and E. coli. Formaldehyde inhibits repair of O6-methylguanine and potentiates the mutagenicity of an alkylating agent, N-methyl-N-nitrosourea, in normal human fibroblasts. Because formaldehyde alone also causes mutations in human cells, formaldehyde may cause genotoxicity by a dual mechanism of directly damaging DNA and also inhibiting repair of mutagenic and carcinogenic DNA lesions caused by other chemical and physical carcinogens. In some experimental studies, repeated exposure to alkylating agents has lead to an increase in O6-methylguanine-DNA alkyltransferase activity, i.e., an adaptive response. We have shown that human bronchial epithelial cells do not adapt and increase their DNA repair capability. This finding has important implications in carcinogenesis caused by low doses of N-nitrosamines. Membrane damage by carcinogens and tumor promoters can also lead to generation of lipid peroxidation aldehydes and indirect DNA damage. We have recently shown that these aldehydes cause extensive DNA damage and inhibit DNA repair in human bronchial epithelial cells.

PHS 6040 (Rev. 1/84)

GPO 914-918

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

Curtis C. Harris	Chief	LHC	NCI
Hans Krokan	Guest Researcher	LHC	NCI

Objectives:

To understand the mechanism of repair of DNA damage by environmental agents in human epithelial tissues and cells and to investigate the genotoxicity of formaldehyde and other tobacco smoke-related aldehydes.

Methods Employed:

Culture of human epithelial and fibroblastic cells; alkaline elution techniques for detection of DNA single strand breaks (SSB) and DNA protein cross-links (DPC); BND cellulose chromatography for measurement of repair replication; ^{3}H-thymidine incorporation in the presence of hydroxyurea for measurement of unscheduled DNA synthesis; isolation of cellular macromolecules, high pressure liquid chromatography. O6-Alkylguanine-DNA alkyltransferase activity found in extracts from a variety of human tissues was characterized and quantitated in three ways: (1) measuring the specific loss of labeled O6-methylguanine (O6-MeGua) from a ^{3}H-methylated DNA substrate, (2) measuring the production of protein containing S-[^{3}H]-methylcysteine during the reaction with this DNA substrate and (3) measuring the formation of [8-^{3}H]-guanine in DNA when the extracts were incubated with a synthetic DNA substrate containing O6-MeGua labeled in the 8-position.

Major Findings:

We have investigated alkyltransferase activity in various human tissues and compared it with the corresponding rat tissues. The alkyltransferase activities of cultured normal human bronchial epithelial cells and fibroblasts were also compared. Extracts of human colon, esophagus, and lung had lower activities than those previously found in human liver samples that showed somewhat higher activities than human brain. When compared with the corresponding rat tissue, human tissue samples contained 2- to 10-fold higher levels of alkyltransferase activity.

Formaldehyde (HCHO) is a common environmental pollutant found in tobacco smoke and a metabolite of demethylation reactions of drugs and carcinogenic N-nitrosamines. It is also a respiratory carcinogen in rats and a potential carcinogenic hazard in humans. Therefore, we have initiated a systematic study of the genotoxicity of HCHO in cultured human cells. The alkaline elution technique was used to study repair of DNA damage caused by HCHO in human bronchial epithelial

cells and fibroblasts, skin fibroblasts, and DNA excision repair-deficient skin fibroblasts from donors with xeroderma pigmentosum (XP). Exposure of cells to HCHO resulted in DNA-protein cross-links (DPC) and DNA single strand breaks (SSB) in all cell types. DPC were induced at similar levels and were also removed by all cell types, including the XP cells. By excision repair of HCHO-induced DNA damage, normal cells generated SSB that were also readily repaired. HCHO was only moderately cytotoxic to normal bronchial epithelial cells and fibroblasts at concentrations that induced substantial DNA damage. HCHO enhanced the cytotoxicity of both ionizing radiation and N-methyl-N-nitrosourea in both cell types. The results indicate that most DPC caused by HCHO can be removed without the involvement of DNA excision repair. Furthermore, HCHO also directly causes DNA SSB as well as SSB generated indirectly during UV-type excision repair. These studies indicate the complexity of the HCHO-induced DNA damage and its repair and that HCHO may enhance the cytotoxicity of chemical and physical carcinogens in human cells.

Since HCHO is formed in equimolar quantities with methylcarbonium ions during the metabolic activation of N-nitrosodimethylamine, we have recently examined the effects of HCHO on the repair of the promutagenic lesion O6-MeGua formed following N-nitrosodimethylamine metabolism. HCHO decreases O6-alkyltransferase activity, inhibits the removal of O6-MeGua, and in low concentrations, synergistically potentiates the cytotoxicity and mutagenicity of N-methyl-N-nitrosourea. In high doses (100 or 130 μM), HCHO is detectably mutagenic itself. Therefore, exposure to HCHO may lead to the dual genotoxic mechanism of both directly damaging DNA and inhibiting repair of mutagenic and carcinogenic lesions caused by alkylating agents and physical carcinogens.

Normal human bronchial epithelial cells cultured in serum-free medium were exposed to low doses of N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) to examine whether increased cellular resistance and increased activity of the DNA repair enzyme O6-methylguanine-DNA methyltransferase could be induced. After treatment with single doses of MNNG a dose-dependent decrease in O6-methylguanine-DNA methyltransferase activity was observed, as expected, for this unique repair system. The activity recovered to the starting level in about 24 hours when a dose that consumed approximately 65% of the enzyme activity (0.2 μg/ml) was given, but did not exceed the activity in the untreated control. Furthermore, treatment every 6 hours for 4 to 5 days with nontoxic concentrations of MNNG (0.04-0.12 μg/ml) did not increase O6-methylguanine-DNA methyltransferase activity. Neither was cell survival following a range of challenge doses significantly increased. Our data suggest that human bronchial epithelial cells do not adapt to MNNG.

Lipid peroxidation aldehydes of the 4-hydroxy-α,β-unsaturated type as well as the tobacco-smoke related α,β-unsaturated aldehyde acrolein were highly cytotoxic and reduced intracellular thiols in cultured human bronchial fibroblasts after treatment with micromolar concentrations. In comparison, formaldehyde and acetaldehyde were less toxic and 100 to 300-fold higher doses

were required to affect survival or thiol levels. The unsaturated aldehydes also inhibited the DNA repair enzyme O6-methylguanine-DNA methyltransferase known to have a cysteine residue in its active site, but had no effect on the activity of uracil-DNA glycosylase. Our results indicate that reactive aldehydes of exogenous or endogenous origin have direct cytotoxic effects and may also make cells more susceptible to other toxic chemicals due to a reduction in cellular defense mechanisms, e.g., DNA repair and detoxification by systems requiring glutathione.

Significance to Biomedical Research and the Program of the Institute:

Methodologies developed for and utilized in studies of DNA damage and repair in animal (normal and tumor) cells can be successfully extended to similar investigations in cells cultured from human tissues susceptible to carcinogenesis. These investigations should aid in identifying mechanisms by which chemical and physical agents will damage the genetic material and exert carcinogenic and/or cocarcinogenic properties.

Proposed Course:

Identify endogenous and exogenous agents, especially those found in tobacco smoke or produced by tumor promoters that will damage DNA and/or affect its repair. To investigate the genotoxicity of acrolein. To determine effects of aldehydes on membrane peroxidation and intracellular calcium. To compare the levels of DNA damage (i.e., DNA SSB, DPC or repair replication) with levels and persistence of DNA adducts caused by chemical carcinogens. To continue correlation of the extent of DNA damage from various agents with biological effects, including toxicity, mutagenesis, and transformation assays.

Publications

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05193-05 LHC

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT (*80 characters or less. Title must fit on one line between the borders.*)

Growth and Differentiation of Normal Human Epithelial Cells and Carcinoma Cells

PRINCIPAL INVESTIGATOR (*List other professional personnel below the Principal Investigator.*) (*Name, title, laboratory, and institute affiliation*)

PI:	Susan P. Banks-Schlegal	Senior Staff Fellow	LHC	NIH
Others:	Curtis C. Harris	Chief	LHC	NIH

COOPERATING UNITS (*if any*)

Litton Bionetics, Rockville, MD (M. Valerio); University of Alabama, Birmington, AL (K. Sexton); University of Maryland (B. Trump, E. McDowell, and T. Wilson)

LAB/BRANCH

Laboratory of Human Carcinogenesis

SECTION

In Vitro Carcinogenesis Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.2	1.2	1.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK (*Use standard unreduced type. Do not exceed the space provided.*)

We have characterized the pattern of expression of the main markers of epithelial differentiation, namely keratins, involucrin and cross-linked envelopes, in human epithelia derived from these major cancer sites during the course of terminal differentiation in embryonic development, in postnatal maturation, and/or in neoplasia. The pattern of expression was found to be dependent on the cell type, the stage of differentiation and/or development and the extrinsic environment of the cell. Distinctive qualitative and quantitative differences in the spectrum of keratin proteins were found in the carcinomas compared with their nontransformed counterparts. Moreover, assessment of cross-linked envelope-forming capabilities or for the presence of involucrin served as specific markers for squamous and urothelial differentiation; the extent of involucrin staining or envelope formation correlated well with the degree of squamous differentiation in both normal epithelia and tumors. We have established human esophageal and lung carcinoma cell lines in cell culture and compared the growth and differentiated properties of these carcinoma cells with their nontransformed counterparts. Significant changes in the array of keratins and in the proportions of cells making cross-linked envelopes were noted. The results we obtained paralleled findings with tumor masses indicating that the tumor cells in cell culture continue to maintain a program of gene expression reflective of that of the original tumor.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on This Project:

Susan P. Banks-Schlegel	Senior Staff Fellow	LHC	NCI
Curtis C. Harris	Chief	LHC	NCI

Objectives:

The growth and differentiation of normal human epithelial cells will be analyzed and compared with those of their neoplastic counterparts. We will attempt to establish neoplastic epithelial cells in culture and examine the growth and differentiated properties of these cells compared with their normal counterparts. These studies are designed to provide insight into the specific changes that occur during malignant transformation. In particular, initial studies will evaluate the usefulness of analysis of keratin protein patterns and cross-linked envelopes in the characterization of epithelial neoplasms. Other defects in the pathway of terminal differentiation will also be assessed.

Methods Employed:

Human tissue is obtained from "immediate" autopsy (i.e., performed within 1 hour of death), from autopsy material that is less than 12 hours postmortem or from surgical specimens. The epithelium and some adherent connective tissue are carefully dissected from the remainder of the tissue. In some instances, the epithelium is separated from the adherent stroma either by heat separation or by surgical excision and the epithelium is analyzed for keratin proteins. In other cases, the epithelium is first cut into explants and used for radiolabeling of proteins. Tumors (minced) were radiolabeled in an analogous manner. When being used for cell culture, the epithelium or tumor is minced and trypsinized to obtain a single-cell suspension. The cells are grown on tissue culture dishes containing a layer of lethally irradiated mouse 3T3 cells. The cells are fed every 3 to 4 days in medium containing 10% fetal calf serum plus various hormonal supplements and growth factors. The morphological and biochemical characterization of the cells is being assessed by a variety of techniques: light and electron microscopy, histochemical staining, immunofluorescent staining, radiolabeling of macromolecules, immunoprecipitation, polyacrylamide gel electrophoresis, autoradiography and peptide mapping. The terminal differentiation of the cells is triggered by means of calcium ionophore and the extent of terminal differentiation is assessed by cross-linked envelope formation. When using human tumor material, the epithelium is not separated from adherent stroma. Otherwise, the tumor and normal tissues are handled identically.

Major Findings:

Cancer of the human esophagus and lung occurs worldwide and frequently represents a major cause of death in the population. Therefore, we have chosen to focus our efforts on these two organ systems.

Human Esophageal Epithelium and Esophageal Carcinoma: Tumor and Cell Culture Studies:

In contrast to the simplified keratin content of bovine, rabbit and rat esophageal epithelium (composed mainly of a 57 and 46 or 51 kD keratin, depending on the animal species), human esophageal epithelium contains a quantitatively different array of keratin proteins, ranging in MW from 37 to 61 kD. The pattern of keratin proteins from human esophageal epithelium differs qualitatively and quantitatively from that of human epidermis. Human esophageal epithelium lacks the 63, 65 and 67 kD keratins characteristic of human epidermis, consistent with the absence of a granular layer and an anucleate stratum corneum. Human esophageal epithelium contains a distinctive 61 kD keratin protein, which was either not present or present in only small amounts in human epidermis, and variable amounts of a 37 kD keratin. Although the 56, 59 and 67 kD keratins were the most abundant keratins in human epidermis, the 52, 57 and 61 kD keratins predominated in human esophageal epithelium. During in vitro cultivation, both human epidermal and esophageal keratinocytes produced colonies that were stratified; however, the morphologic appearance of these cultured epithelia differed. Only cultured human epidermal keratinocytes contained keratohyalin granules in the outermost layers and a prominant 67 kD keratin on immunoprecipitation, reflective of their in vivo program of differentiation. Otherwise, the keratin contents appeared similar. In conclusion, human esophageal epithelium exhibited intertissue and interspecies differences in the pattern of keratin proteins. During in vitro cultivation, human esophageal keratinocytes retained some aspects of their distinctive program of differentiation.

Other laboratories have shown that changes in keratin protein synthesis occur during the course of terminal differentiation as the epidermal cell matures during its migration through the spinous layer and finally into the stratum corneum layer. The inner layers of epidermis contained small keratins and some amounts of large keratin and the outer layers contained predominantly larger keratins in addition to the small ones. Using antibodies made to individual SDS-gel purified keratins and immunohistochemical staining techniques, we defined the precise topographical distribution of several keratin proteins and their relationship to the process of epidermal differentiation. The two smallest keratin proteins (45 kD and 46 kD) localized predominantly in the basal cells, whereas the higher MW keratin (55 kD and 63 kD) were found chiefly in the overlying differentiated keratinocytes. With the exception of the lack of a granular layer and an anucleate stratum corneum, human esophageal epithelium and epidermis are morphologically similar. To examine for similar shifts in keratin protein synthesis during the course of terminal differentiation in human esophageal epithelium, we stained sections of human esophagus using antibodies to the individual keratins and the immunoperoxidase staining technique. Surprisingly, in contrast to the two distinct keratin domains found in human epidermis, human esophageal epithelium exhibited no definitive localization of specific keratins either to the proliferative or differentiated cellular components. We found both lower and higher MW keratins to be distributed fairly uniformly throughout the entire epithelium. Additional evidence in support of these findings came from studies in which we made serial parallel sections through the human esophageal epithelium and examined the pattern of keratins extracted from these slices on SDS-polyacrylamide gels. In comparison to human epidermis which showed shifts from

small to large keratin during the course of epidermal differentiation, only quantitative changes in the keratin profile were noted in human esophageal epithelium during the course of terminal differentiation, consistent with the antibody staining data. In conclusion, despite the similarities in morphological differentiation, the programs of biochemical differentiation are very distinctive between human esophageal epithelium and epidermis. Human esophageal epithelium did not exhibit the sharp transition from the low MW keratins (45 and 46 kD) to higher MW keratins (55 and 63 kD) which in epidermis paralleled the loss of cell proliferation potential and the commitment of the basal epidermal cell to terminal differentiation.

Biochemical as well as immunological approaches investigating changes in keratins during the course of terminal differentiation in adult human esophageal epithelium indicated that the program of biochemical differentiation of human esophageal epithelium was not accompanied by dramatic qualitative shifts in keratins during the course of terminal differentiation as had been observed with epidermis. Since previous studies from this laboratory have shown that the changes which occur during embryonic development are a presage of adult epithelial maturation, we examined rabbit esophageal epithelium for changes in keratin expression during embryonic development. In contrast to the dramatic qualitative and quantitative alterations in keratins, observed during embryonic epidermal development, no significant qualitative changes in the keratins were observed during terminal differentiation of human esophageal epithelium throughout the course of embryonic esophageal development. There did appear to be some quantitative increases in the keratins during the course of embryonic development, these increases being somewhat more pronounced in the case of the small keratins (46-50 kD). The 44 kD keratin appeared to decrease in concentration during development. Similarly, keratins extracted from rabbit esophageal keratinocytes grown from embryonic esophageal epithelium at early and late stages of embryonic development exhibited similar protein profiles. These data support the earlier findings that despite similarities in their program of morphological differentiation, esophageal epithelium differs markedly from epidermis in terms of its program of keratin expression both during the course of terminal differentiation during embryonic development and during the sequential maturation steps of the adult epithelium.

Analysis of keratin proteins extracted from human esophageal tumors revealed dramatic changes in the pattern of keratins. In addition to an overall reduction in the amount of keratin, most tumors were characterized by a complete loss of the major 52 and 61 kD esophageal keratins. The lower MW keratins (48 to 50.5 kD) and the 57 kD keratin were conserved in the transformed phenotype. Injection of these tumors into nude mice and analysis of the tumors for keratin proteins revealed an even more dramatic shift in patterns of keratins. To analyze for alterations in the pathway of terminal differentiation in human esophageal carcinomas, we examined for the expression of another differentiated function, cross-linked envelopes. The formation of cross-linked envelopes, structures resistant to SDS and a reducing agent, was induced using the calcium ionophore, X-537A, and then the extent of terminal differentiation was estimated by calculating the percentage of cells that formed cross-linked envelopes. Compared to normal esophageal epithelial cells, human esophageal carcinoma cells exhibited a variable capacity to form cross-linked envelopes, ranging from unimpaired to a severely restricted capacity to form cross-linked envelopes. In general, there was some correlation between envelope-forming capabilities and the degree of tumor differentiation with more differentiated tumors forming more envelopes.

While the majority of the tumors examined (6 out of 8 cases) displayed a markedly reduced capacity to form envelopes, indicating a defect in the pathway of terminal differentiation, carcinoma cells from two tumors formed cross-linked envelopes at levels comparable to that of normal esophageal epithelium.

After repeated attempts at establishing esophageal epithelial tumors in culture, we have succeeded in establishing seven tumor cell lines. We have undertaken a number of studies to examine various growth and differentiated properties exhibited by these human esophageal carcinoma cells. While the cells from most of the tumor cell lines looked typically epithelial, the cells were morphologically distinguishable from normal human esophageal epithelial cells when examined by phase contrast microscopy. In contrast to normal esophageal epithelial cells, which were uniform in appearance and polygonally shaped and underwent a very organized and orderly stratification process, human esophageal carcinoma cells were very pleomorphic, varied greatly in size and shape and tended to pile up on one another in an unorganized manner. When grown under optimal growth conditions (Medium 199 containing 10% fetal calf serum and various growth supplements), human esophageal carcinoma cells reached a much higher saturation density (5 to 8 x 10^6 cells/60 mm dish) than their nontransformed counterparts (2.5 to 3 x 10^6 cells/60 mm dish). Their doubling times ranged from approximately 33 hours (similar to normal human esophageal epithelial cells) to 82 hours. Because transformed cells have been reported to exhibit reduced serum and growth factor requirements, we also examined the ability of the cells to grow under more stringent growth conditions (Medium 199 plus 2% fetal calf serum and hydrocortisone). Interestingly, although the carcinoma cells grew better than the normal cells under these growth conditions, they grew much more slowly than they had under optimal growth conditions (in most cases) and reached lower saturation densities (only 1 to 1.5 x 10^6 cells/60 mm dish), suggesting that these esophageal carcinoma cells are not significantly altered in terms of their serum and growth factor requirements compared to their nontransformed counterparts. Similar to normal esophageal epithelial cells, the esophageal carcinoma cell still required cocultivation with a layer of irradiated 3T3 fibroblasts for growth at clonal densities. When assessed for anchorage-independent growth, all carcinoma cells displayed some capacity to grow in agarose, although the colony-forming efficiency and size of the colonies varied, depending on the cell line. Normal esophageal epithelial cells did not form colonies in agarose. When tumorigenicity in nude mice was evaluated, the different esophageal carcinoma cell lines also exhibited varying capacities to form tumors in nude mice.

Next, we investigated human esophageal carcinoma cell lines for the expression of certain differentiated functions associated with normal human esophageal epithelial maturation, namely keratin proteins, a major cytoskeletal component and cross-linked envelopes as a measure of the extent of terminal differentiation. Radiolabeled keratin proteins were extracted using high salt and detergent, selectively immunoprecipitated with keratin antiserum, and analyzed on gels. Surprisingly, in contrast to the results with tumor masses (summarized above) in which specific changes in keratin proteins characterized the malignant phenotype, the esophageal carcinoma cell lines exhibited a greater variability in the spectrum of keratin proteins associated with malignant transformation. While some cell lines exhibited the loss of the major 52 kD keratin, others did not. Moreover, some cell lines showed a loss of the 57 kD keratin, which was never found to be missing in studies with esophageal tumor masses, and two lines expressed a high MW 67 kD keratin. Human esophageal tumors in nude mice always revealed

loss of the 61 and 52 kD keratins, similar to tumor masses. The patterns of keratin proteins in tumors formed by injection of the carcinoma cell lines into nude mice revealed there was concerted modulation in the expression of a 44 kD keratin, a 52 kD keratin and a 67 kD keratin in two cell lines (HLE-4 and HCE-6); specifically, the synthesis of the 44 and 52 kD keratins was suppressed coincident with the appearance of the 67 kD keratin in tumors derived from these cell lines. These keratin patterns were once again reversed in cell lines recultured from these tumors, suggesting that the expression of these specific keratins is subject to extrinsic growth regulation. In contrast to the typical perinuclear arrangement of keratin filaments found within normal esophageal epithelial cells, keratin filaments in esophageal carcinoma cells were usually distributed uniformly throughout the cytoplasm or located peripherally, frequently in association with desmosomes. To assess the extent of terminal differentiation in cultured normal human esophageal epithelial cells and eophageal carcinoma cells, cells were induced to terminally differentiate with calcium ionophore and then the percentage of cells with cross-linked envelopes, structures resistant to detergent and a reducing agent, was determined. Analogous to findings with tumor masses, some, but not all, esophageal carcinoma cell lines were found to exhibit a reduced capacity to form cross-linked envelopes. Therefore, these results suggest that expression of the transformed phenotype sometimes, but not always, leads to a defect in the pathway of terminal differentiation as assayed by this marker. These cell lines are currently being examined for alterations in the expression of a number of other properties commonly ascribed to transformation.

Retroviruses (RNA tumor viruses) contain segments of cellular DNA that have been implicated in the induction of neoplasia. These segments have been referred to as viral oncogenes (v-onc). DNA sequences homologous to these genes (termed cellular oncogenes or c-onc) have been identified in normal cells and in some cases their ability to induce malignancy has been shown. Associations between these oncogenes and human malignancy have been shown and in some cases where it was possible to study normal and malignant tissue from the same organ and patient, transcriptional activity of certain oncogenes was found to be enhanced in the malignant tissue.

We attempted to transform human esophageal epithelial cells in cell culture using various oncogenes and oncogene combinations. The oncogenes which were tested were the following: v-ras^{Ha}, v-ras^{Ki}, v-abl, v-raf, v-myc, Ela and pSV2neo containing Ela, v-raf, raf-myc and myc (Burkitt's lymphoma). In some cases v-ras^{Ha} was co-transfected with myc or Ela. DNA was introduced into cells using either protoplast fusion or electro-shock methods. Initially, the main parameter being assayed for was immortality. In the cases of experiments utilizing constructs containing a neo gene, possible transformants on half of the experimental dishes were first selected from the total population by growth of the cells in medium containing G418. The remaining dishes were selected for on the basis of immortality. Control dishes receiving either salmon sperm DNA alone or pSV2neo alone were treated identically to the experimentals. In no case were neo-resistant cells or immortalized cells detected.

Human Bronchial Epithelium and Bronchiogenic Carcinomas: Tumor and Cell Culture Studies

Most human lung tumors arise from the area of the bronchus. They have been classified by the World Health Organization on the basis of their histological

appearance and synthetic product(s) into four major classes, specifically squamous cell carcinoma, adenocarcinoma, large cell carcinoma and small cell lung carcinoma. Keratins have been found to be useful not only for delineating the epithelial nature of the tumor but also as an adjunct in defining the type of tumor present. For instance, well-differentiated squamous cell carcinomas, urotheliomas and mesotheliomas tend to be strongly keratin positive. Adenocarcinomas tend to be weakly positive to negative. Both adenocarcinomas and squamous cell carcinomas of the lung contained keratin proteins, as demonstrated by immunocytochemical and electron microscopic data. However, the amount of keratin varied depending on the tumor type (decreased in adenocarcinomas) and the degree of squamous differentiation (decreased in poorly differentiated tumors) (studies performed in collaboration with Drs. Elizabeth McDowell, Benjamin Trump and Tom Wilson at the University of Maryland). Since the lung tumors were not easily classified on the basis of immunoperoxidase staining or ultrastructural localization of keratin, we analyzed keratin-enriched protein fractions of these tumors by one-dimensional gel electrophoresis to investigate their usefulness in distinguishing these lung neoplasms. Keratin extraction data and keratin immunoprecipitation data revealed that there were distinct qualitative and quantitative differences useful in distinguishing adenocarcinomas from squamous cell carcinomas of the lung.

Human lung tumor cell lines established from the major histological types of lung cancer were examined by immunofluorescent staining techniques for their pattern of intermediate filament (IF) (keratin, vimentin and neurofilament triplet protein) expression. The cell lines had been established by Dr. Adi Gazdar and collaborators at the NCI Medical Oncology Branch, Bethesda, MD. All cell lines examined, both small cell lung carcinoma (SCLC) and non-SCLC (squamous cell carcinoma [SQC], adenocarcinoma [AC], large cell carcinoma [LCC] and mesothelioma) contained keratin, consistent with their epithelial derivation. These lung carcinoma cell lines also expressed vimentin, the characteristic intermediate filament of mesenchymal cells in vivo, similar to previous reports demonstrating a coexpression of vimentin and keratin in carcinoma cells in vitro. In light of the proposed "neuroectodermal" origin of SCLC, cell lines were also studied for neurofilament expression. Two of four SCLC tumor cell lines, as well as non-SCLC cell lines, showed no reactivity with antibodies to neurofilament triplet protein. Two of the SCLC cell lines stained weakly with anti-neurofilament antibody. Examination of specific keratin patterns in human lung tumor cell lines by selective immunoprecipitation with keratin antiserum and SDS-polyacrylamide gel electrophoresis indicated that low MW forms of keratin protein (44 to 52 kD) were present in cell lines derived from SCLC and non-SCLC types of lung cancer. Tumor cell lines exhibited squamous differentiation (by light microscopic criteria) also displayed a prepondrance of intermediate MW forms of keratin (57 and 59 kD) and exhibited another feature of terminal keratinocyte differentiation (cross-linked envelope formation). Mesothelioma cell lines had varying keratin profiles, perhaps related to their pleomorphic nature and the simultaneous expression of two types of IF in vivo, the relative amounts of which correlate with their morphologic appearance. The keratin profiles of the lung cancer cells provide further evidence of a biochemical link between SCLC and non-SCLC types of bronchogenic carcinoma.

The biochemical characterizations of the various epithelia and tumors have been compatible with immunological approaches using specific antibodies, as demonstrated in studies performed in collaboration with Dr. J. Said at Cedars-Sinai Medical Center and Drs. G. Pinkus and J. Corson at Harvard Medical School.

Involucrin and Cross-Linked Envelopes: Specific Markers of Squamous and Urothelial Differentiation. Distribution in Normal and Neoplastic Tissues

In a previous study we have shown that different stratified squamous epithelia, whether they possess an anucleate stratum corneum, posseses the precursor protein of the cross-linked envelope that is characteristic of epidermal stratum corneum and can be induced to form cross-linked envelopes. This protein, involucrin, first appears in cells located in the outer 1/2 to 1/3 of the epithelium. In cultured epithelia, involucrin first appears in cells immediately above the basal layer, presumably due to accelerated terminal differentiation. Studies from other laboratories have shown that the appearance of mRNA for involucrin and synthesis of the protein is an orderly function of normal terminal maturation, which correlates with the size of keratinocytes and their level within the epidermis. Hence, both involucrin and cross-linked envelope formation appear to serve as markers of terminal keratinocyte differentiation. In collaboration with Dr. Jonathan Said at Cedars-Sinai Medical Center we have assessed the distribution of involucrin in a wide variety of normal and neoplastic tissues. With few exceptions and in contrast to keratins which are present in most, if not all, epithelia, involucrin was restricted to squamous epithelia, urothelium, some skin appendages and thymic Hassall's corpuscles. All other normal tissues studied were negative. In normal squamous epithelium and normal urothelium, staining was most intense in the superficial cell layers where it was concentrated at the cell periphery and gradually decreased toward the basal layer. The orderly staining pattern was maintained in benign squamous and urothelial lesions and in grade I papillary urothelial carcinomas. Higher grade papillary urothelial carcinomas, infiltrating urothelial and squamous carcinomas and in situ urothelial and squamous carcinomas demonstrated abnormal staining patterns for involucrin. In situ carcinomas and higher grade papillary carcinomas showed intense staining limited to individual larger cells scattered throughout the epithelium in contrast to the orderly gradation of staining present in the normal epithelium possibly representing focal inappropriate or premature terminal differentiation and suggestive of a loss of differentiation. Similar staining patterns have been observed in Bowen's disease and squamous carcinoma in situ of skin, vulva and vagina. On the other hand, well-differentiated infiltrating squamous carcinomas of the larnyx, nasopharynx, esophagus, vagina, vulva, urinary bladder and skin showed abundant staining for involucrin often limited to larger cells in the more differentiated areas of the tumors while poorly differentiated tumors and basal cell carcinomas of the skin were either negative or stained only in larger tumor cells or in areas of squamous differentiation. Similar results were found for infiltrating squamous carcinomas of the lung. Hence, differentiated areas of in situ and invasive squamous carcinomas contained involucrin. Foci of squamous differentiation in adenocarcinomas and other epithelial malignancies stained intensely for involucrin. Brenner tumors of the ovary and Walthard rests of the fallopian tube, which are lesions of uncertain histogenesis but possibly urothelial-related, also stained for involucrin, supporting their derivation either from coelomic epithelium by a process of urothelial metaplasia or from urogenital rests. The results of these studies

suggest that (1) involucrin is a sensitive and specific marker for squamous and urothelial differentiation; (2) staining patterns for involucrin may be helpful in distinguishing benign from malignant urothelial and squamous lesions.

Of interest were the abnormal patterns of increased staining for involucrin deep into the lower spinous cell layers of the epidermis adjacent to squamous cell carcinomas and overlying basal cell carcinomas. In this regard, abnormal patterns of keratin staining in these histologically normal-appearing areas of epidermis were also noted with low MW keratins (45 and 46 kD) staining, diffusely, all layers of the epidermis (usually a predominantly basal staining pattern) and showing decreased intensity of suprabasal staining for high MW keratin (63 kD). These altered patterns of involucrin and keratin staining were not specific for neoplasia, but were also encountered in various benign epidermal hyperplasias. These changes could be due to abnormal squamous maturation, increased rate of cell turnover, response of the adjacent epidermis to the tumor microenvironment or other factors.

Measurement of the ability of a cell to form cross-linked envelopes, similarly serves as a specific marker for squamous differentiation. Assessment of cross-linked envelope expression in human esophageal carcinomas, esophageal carcinoma cell lines and human lung tumor cell lines demonstrated that only cells exhibiting squamous differentiation at the light microscopic level exhibited a capacity to form envelopes following induction of envelope formation with the calcium ionophore, X-537A. A tumor cell's capacity to form envelopes as well as involucrin correlated largely with the extent of tumor differentiation and is consistent with studies from other laboratories demonstrating a correlation between involucrin synthesis or envelope formation and cell size and stage of differentiation. Both involucrin and cross-linked envelopes represented differentiation-specific antigens present in larger cells in the center of squamous cell nests and absent in the small basal oriented cells at the periphery of tumor nests. Most, but not all, tumor cells exhibited an impaired capacity to form envelopes. Such a defect in the triggering of terminal differentiation could augment clonal expansion by a malignant cell.

Significance to Biomedical Research and the Program of the Institute:

Most human cancers are epithelial in origin. A better understanding of the complex process of neoplasia will require both a full understanding of the normal program of differentiation in human epithelial cells and how it is altered during malignant transformation. Advances in the ability to grow human epithelial cells in culture will undoubtedly facilitate attempts to unravel the mechanism(s) involved in malignant transformation.

Proposed Course:

Studies aimed at understanding the control of differentiation and the sequence of events involved in malignant transformation of epithelial cells will continue.

Publications

Banks-Schlegel, S. P., Gazdar, A. F. and Harris, C. C.: Intermediate filament and cross-linked envelope expression in human lung tumor cell lines. Cancer Res. 45: 1187-1197, 1985.

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Sassoon, A. F., Said, J. W., Nash, G., Shintaku, I. P. and Banks-Schlegel, S. P.: Involucrin in intraepithelial and invasive squamous cell carcinomas of the cervix. An immunohistochemical study. Human Pathol. (In Press)

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05291-04 LHC

PERIOD COVERED

October 1, 1984 to Sepetember 30, 1985

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

DNA Adducts in People Exposed to Polycyclic Aromatic Hydrocarbons

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	Curtis C. Harris	Chief	LHC	NCI
Others:	Kirsi Vahakangas	Visiting Associate	LHC	NCI
	Dean L. Mann	Section Chief	LHC	NCI
	Glennwood E. Trivers	Biologist	LHC	NCI
	Ainsley Weston	Visiting Fellow	LHC	NCI

COOPERATING UNITS (if any) Mt. Sinai School of Medicine, New York, NY (R.R. Boesch); Univ. of MD School of Medicine, Baltimore, MD (B.F. Trump); Georgetown Univ. School of Medicine, Washington, DC (H. Yeager); Univ. of California School of Medicine, Los Angeles, CA (W. Wright)

LAB/BRANCH

Laboratory of Human Carcinogenesis

SECTION

Biochemical Epidemiology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.0	2.0	

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Benzo[a]pyrene (BP) is a ubiquitous carcinogen found in tobacco smoke, burning of fossil fuels, and our diet. Formation of BP diol epoxide (BPDE)-DNA adducts due to human exposure is most likely to be at very low levels that are beyond the sensitivity of routine radioimmunoassay and chromatographic analyses. Thus, ultrasensitive enzymatic radioimmunoassay (USERIA), enzyme-linked immunosorbent assay (ELISA), and synchronous scanning fluorimetry have been employed to detect and quantitate BPDE-DNA adducts in humans at high cancer risk due in part to BP exposure. DNA isolated from white blood cells of asphalt workers (roofers) and foundry workers and DNA from lung tissue, bronchial washings, and alveolar macrophages of lung cancer patients and smokers are being investigated. Putative BPDE-DNA adducts have been detected in several of the high-risk individuals. Antibodies to BPDE-DNA adducts were also found in sera from those workers. These data suggest that the activation of BP to its ultimate carcinogen as well as formation of adducts with DNA occurs in humans. Preliminary evidence indicates that other polycyclic aromatic hydrocarbons (e.g., chrysene and benzo(a)anthracene, DNA adducts) may also evoke an immune response in humans.

PHS 6040 (Rev. 1/84)

PROJECT DESCRIPTION

Names Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

Curtis Harris	Chief	LHC	NCI
Kirsi Vahakangas	Visiting Associate	LHC	NCI
Dean L. Mann	Section Chief	LHC	NCI
Glennwood E. Trivers	Biologist	LHC	NCI
Ainsley Weston	Visiting Fellow	LHC	NCI

Objectives:

Using rabbit anti-benzo[a]pyrene diol epoxide (BPDE) antibodies and the most sensitive immunoassays available, BPDE-DNA adducts and human antibodies to these adducts will be determined in high-risk individuals. Results should help us in further understanding activation and mechanism of carcinogenesis in humans.

Methods Employed:

Twenty-five to 40 ml of peripheral blood was obtained from 28 male volunteers who were active in their occupation as roofers for over 20 years. The blood samples were centrifuged at 100 x g for 15 minutes, and the "buffy coat" was homogenized in 5 volumes of HKM:0.25 M sucrose buffer (0.05 M HEPES, pH 7.3; 0.024 M (KCl; 0.05 M $MgCl_2$) using a glass homogenizer. The homogenate was centrifuged for 10 minutes at 300 x g at 4°C. The pellet was suspended in HKM-sucrose buffer containing 0.5% Triton 100 and centrifuged for 10 minutes at 4°C. The pellet was suspended in HKM-sucrose buffer and recentrifuged. The final pellet was resuspended in 5 ml HKM-sucrose buffer containing 1% SDS and 1 M NaCl. An equal volume of chloroform-isoamyl alcohol (24:1, v/v) was added, and the mixture was vigorously agitated for at least 20 minutes followed by centrifugation at 10,000 x g for 10 minutes. The aqueous epiphase was removed by winding onto a glass rod. Residual ethanol was removed by nitrogen and DNA dissolved in water. Purity and quantitation of DNA were determined by absorbance at 260 nm and 280 nm using a Beckman DU8 spectrophotometer and a fluorometer. The final volume of DNA solution was adjusted to 1 mg DNA/ml water, and the solution was rendered single-stranded by boiling. Single-stranded DNA was then stored at 4°C until tested. DNA was similarly isolated from lung tissue, bronchial washings and alveolar macrophages. DNA from foundry workers was received in purified form, ready to be tested.

Competitive enzyme immunoassays, USERIA and ELISA, were performed on the test DNA samples by using rabbit anti-BPDE-DNA antibody. Polyvinyl U-bottom 96-well microtiter plates (Dynatech Laboratories, Inc., Alexandria, VA) were coated with unmodified DNA (control) and BPDE-modified DNA (1 ng/well for USERIA and 5 ng/well for ELISA). Standard competitive inhibition curves were obtained by mixing serial dilutions of known standard BPDE-DNA with rabbit antisera. Percentage inhibition of the test samples was determined from the standard curves. All tests and assays were done in duplicate, and the standard deviation was less than 10%.

Major Findings:

Coke oven workers are exposed to high levels of carcinogenic polycyclic aromatic hydrocarbons, including benzo[a]pyrene (BP), and are at increased risk of lung cancer. Since BP is enzymatically activated to 7β,8α-dihydroxy-(9α,10α)-epoxy-7,8,9,10-tetrahydro BP (BPDE) that forms adducts with DNA, the presence of these adducts was measured in DNA from peripheral blood lymphocytes by synchronous fluorescence spectrophotometry and enzyme radioimmunoassay. Approximately two-thirds of the workers had detectable levels of BPDE-DNA adducts. Antibodies to the DNA adducts were also found in the sera of 27% of the workers. Human sera reacting with BPDE-DNA may contain antibodies that also recognize chrysene-modified DNA. These antigens may share a commone epitope or the polyclonal human sera may contain antibodies specific for each antigen. DNA adducts and/or antibodies to the adducts indicate exposure to BP and its metabolic activation to the carcinogenic metabolite than covalently binds to and damages DNA. Detection of adducts and antibodies to them may also be useful as internal dosimeters of the pathobiological effective doses of chemical carcinogens.

Significance to Biomedical Research and the Program of the Institute:

Demonstration of carcinogen-DNA interaction in human tissue will enable us to better understand the mechanism of carcinogenesis in humans. Although white blood cells may not be the prime target for certain carcinogens, the presence of carcinogen-DNA antigenicity in these cells not only suggests a widespread distribution of the carcinogen but also provides an opportunity to screen high-risk individuals with relatively simple procedures. Antibodies to carcinogen-DNA adducts may be useful as an indicator of past exposure and metabolic activation of carcinogens.

Proposed Course:

Since BPDE-DNA antigenicity suggests the presence of BPDE-DNA adducts in humans, we are in the process of further documenting this result using biophysical approaches to measure carcinogen-DNA adducts. Anti-carcinogen-DNA antibodies found in donors who are exposed to polycyclic aromatic hydrocarbons will also be characterized as to immunoglobulin type and to specificity. Sera will also be analyzed for antibodies to DNA adducts formed by other chemical carcinogens found in tobacco smoke. Human monoclonal antibodies to carcinogen-DNA adducts will be produced using human hybridoma technology. Biochemical and molecular epidemiological studies of high-risk individuals are planned.

Publications

Gabrielson, E. and Harris, C. C.: Carcinogenic research using cultured human tissues and cells. Eur. J. Oncol. Cancer Res. (In Press)

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Harris, C. C., Vahakangas, K., Newman, M., Trivers, G. E., Mann, D. L. and Wright, W.: Detection of benzo[a]pyrene diol epoxide-DNA adducts in peripheral blood lymphocytes and antibodies to the adducts in sera from coke oven workers. Proc. Natl. Acad. Sci. (In Press)

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05293-04 LHC

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Oncogene Transfection of Human Cells

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	George H. Yoakum	Senior Staff Fellow	LHC	NCI
Others:	Paul Amstad	Visiting Fellow	LHC	NCI
	Dimitrios Boumpas	Visiting Fellow	LHC	NCI
	Curtis C. Harris	Chief	LHC	NCI

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Human Carcinogenesis

SECTION

Carcinogen Macromolecular Interaction Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.0	1.5	0.5

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Genetic studies of human cell DNA repair and carcinogenesis have been initiated by protoplast fusion transfection of a variety of types of human cells with specific cellular and viral genes known to play a role in human carcinogenesis. The role of the ras gene in normal human bronchial epithelial cell carcinogenesis is being studied by analyzing the progression of Harvey murine sarcoma virus (v-Ha-ras)transfected HBE cells through the states of resistance to squamous cell differentiation, immortalization, anchorage independent growth, tumorigenicity and metastasis in athymic nude mice. The advantages of genetic transfection vis-a-vis virus/helper-virus experiments to study the biological activities of viral genes includes the ability to produce virus-free human cell lines that stably carry and express virus gene products. This circumvents the biohazards associated with virus shedding cell cultures and the technical problems of human cells releasing transforming viruses during xenotransplantation experiments testing the tumorigenicity of transformed human cells. Characterization of v-Ha-ras-transfected human bronchial epithelial cells, including restriction mapping of transfection loci, selection of tumorigenic and nontumorigenic clones, and determination of conditions required for expression of tumorigenic phenotypes will provide information to elucidate the mechanism of ras-mediated carcinogenesis in an important progenitor cell of human lung cancer.

PHS 6040 (Rev. 1/84)

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

George H. Yoakum	Senior Staff Fellow	LHC	NCI
Paul Amstad	Visiting Fellow	LHC	NCI
Dimitrois Boumpas	Visiting Fellow	LHC	NCI
Curtis C. Harris	Chief	LHC	NCI

Objectives:

The primary goals of this research project are development and application of a genetic approach to problems of human carcinogenesis at the molecular level. This research program focuses on the genetic role of Ha-ras in normal human bronchial epithelial cell (NHBE) transformation by transfection of MSV-Ha ras into human bronchial epithelial (HBE) cells, and the mechansism of biological responses involved in the carcinogenic processes following oncogene transfection.

Methods Employed:

We have developed a method to transfect a variety of human cell types, normal fibroblastic and epithelial cells, i.e., carcinoma cells and transformed fibroblasts. Introduction of exogenous genes (human or viral) to human cells is essential to development of effective research programs in human carcinogenesis at the genetic and molecular levels. This permits the construction of human cell lines from normal human cells carrying oncogenes for characterization of in vitro carcinogenic potential. The human Ha-ras+ tester cells have been developed for carcinogenesis studies (TBE-1). The protoplast fusion method of transfection for transfer of plasmids stably transfers genes into human cells at frequencies greater than 10^{-3} units. We are transfection-testing the following potential oncogene constructs on pSV2 neo-plasmids: (1) v-Ha-ras, (2) v-myc, (3) transfection into normal human bronchial epithelial cells, cord blood lymphoyctes and TBE-1 (HBE with v-Ha-ras+).

Standard nucleic acid hybridization analysis methods will be utilized to characterize the genetic organiztion of human recombinant cell lines constructed for these studies. This includes slot-blot DNA or RNA hybridization to detect the presence and expression of transcripts in human cell transfectants and Southern hybridization of restriction digested nuclear DNA after gel electrophoresis and transfer to nitrocellulose to map genomic DNA inserts and characterize transfected gene structures.

Biological analysis of human recombinants constructed for carcinogenesis studies will employ (1) standard tissue culture methods to determine the culture longevity, growth rate, production of autogenous growth factor(s), and anchorage

independent growth (soft agar growth); (2) tumorigenicity assays by xenotransplantation in athymic nude mice; (3) determination of karyotypic status, isozyme phenotype, immunocytochemical staining for keratin, human chorionic growth hormone, and analysis of surface antigens; and (4) determination of the effects of oncogene expression on DNA repair processes.

Major Findings:

Transfection of NHBE cells with a plasmid carrying the ras oncogene of Harvey murine sarcoma virus (v-Ha-ras) changed the growth requirements, terminal differentiation, and tumorigenicity of the recipient cells. One of the cell lines isolated after transfection (TBE-1) was studied extensively and shown to contain v-Ha-ras DNA. Total cellular RNA from TBE-1 cells hybridized to v-Ha-ras structural gene fragment probes five to eight times more than RNA from parental NHBE cells. The TBE-1 cells expressed phosphorylated v-Ha-ras polypeptide p21, showed a reduced requirement for growth factor supplements, and became aneuploid as an early cellular response to v-Ha-ras expression. As the transfectants acquire an indefinite lifespan and anchorage independence they became transplantable tumor cells with the capability of metastases to the liver, spleen, and lungs and showed many phenotype changes suggesting a pleiotrophic mechansism for the role of Ha-ras in human carcinogenesis.

Selection of v-Ha ras transfectants the cells were cultured in LHC-4 medium with 2 percent BDS for 9 days. Approximately 4 x 10^6 NHBE cells were transfected at 70 to 80 percent confluence in 60-mm dishes by protoplast fusion with a strain of Escherichia coli carrying plasmid H1.A complete transforming sequence form the 5' side of the Ha-MuSV is carried on H1. This selection method yielded v-Ha-ras transfected colonies at a frequency of approximately 10^{-3}.

We tested TBE-1 cultures to determine their growth characteristics, response to TPA, ability to form colonies of anchorage independent cells in soft agar, and tumorigenicity including transplantability and metastasis in athymic nude mice. The results indicated that TBE-1 cells (i) are not induced to squamous terminal differentiation by either BDS or 10^{-7} M TPA; (ii) do not produce a growth factor recognized by normal rat kidney (NRK) cells; (iii) do form colonies in soft agar; and (iv) do produce a TBE-1 growth factor, since population doublings per day increased by a factor of 5.0 when autogenously conditioned medium was used to supplement cell growth at clonal density.

The TBE-1SA cells were isolated by selection of anchorage independent cells growing in soft agar cultures of TBE-1 cells. The progression of this subpopulation of cells was indicated by their ability to form tumors that grew larger than 1.0 cm in diameter and persisted longer than 90 days in 13 of 14 athymic nude mice. These tumors reached 2.5 cm in diameter, did not show the regression that was frequently observed when TBE-1 cells were tested for tumorigenicity, and metastasized after transfer to a secondary recipient. The low frequency and extended latent period observed for TBE-1 tumorigenicity indicates that the selection of

anchorage independent cells (TBE-1SA) yielded a subpopulation of TBE-1 that had progressed to tumorigenicity. The tumorigenicity of the TBE-1SA cells was also indicated by the growth of secondary transplants of TBE-1SA tumor tissue in nude mice.

The human isoenzymes found in TBE-1 were human, lactate dehydrogenase; glucose-6phosphate dehydrogenase; phosphoglucomutase 3; esterase D; mitochondrial malic enzyme; adenylate kinase 1 (soluble); and glyoxalase-1. The matching isoenzyme phenotype of TBE-1, TBE-1SA, and TBE-1SAT cells indicated that in each case the cells were of human origin and that they were the progeny of one donor.

Significance to Biomedical Research and the Program of the Institute:

The transformation of NHBE cells by transfection with v-Ha-ras oncogene provides the first normal human epithelial cell line that has been transformed to malignancy. The use of this procedure to isolate v-Ha-ras oncogene-transfected human bronchial epithelial recombinant cell lines with known tumorigenic potential provides a unique opportunity to study Ha-ras oncogenesis in vitro in an important human progenitor cell. In addition to the mechanistic information provided about the role of Ha-ras in human cell carcinogenesis, these experiments provide the means for in vitro construction of virus-free human cell lines capable of producing biologically active oncogene products for biomedical applications.

Proposed Course:

Oncogene transfection of normal primary human cultures will be used to study the role of other oncogenes in normal human cells in vitro. The oncogenic role of v-Ha-ras will be determined by characterization of the transfected recombinant cell line with assayable tumorigenic potential and by the interaction of v-Ha-ras with other oncogenes. Karyotypic stability and surface marker alterations will be studied after v-Ha-ras transfection of NHBE cells to elucidate the mechanism of Ha-ras oncogenesis. We will also test the episomal DNA from TBE-1 carrying v-Ha-ras for increased transforming activity by transfection into NHBE cells.

Publications:

Yoakum, G. H.: Protoplast fusion: A method to transfect human cells for gene isolation, oncogene testing and construction of specialized cell lines. Bio-Techniques 2: 24-30, 1984.

Yoakum, G. H., Lechner, J. F., Gabrielson, E. W., Korba, B. E., Malan-Shibley, L., Willey, J. C., Valerio, M. G., Shamsuddin, A. M., Trump, B. F., and Harris, C. C.: Transformation of human bronchial epithelial cells transfected by Harvey ras oncogene. Science 227: 1174-1179, 1985.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05324-03 LHC

PERIOD COVERED

October 1, 1984 to Sepetember 30, 1985

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Human Lung Carcinoma/Bronchial Epithelial Cell Hybrid Genetics

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	Edward W. Gabrielson	Medical Staff Fellow	LHC	NCI
Others:	Curtis C. Harris	Chief	LHC	NCI

COOPERATING UNITS (if any)

Centers for Disease Control, Atlanta, GA (J. Lechner)

LAB/BRANCH

Laboratory of Human Carcinogenesis

SECTION

In Vitro Carcinogenesis Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.0	1.0	1.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Genetic changes related to carcinogenesis are being studied using hybrids of human lung carcinoma cells with normal human bronchial epithelial cells. Initial studies suggest that a limited population doubling potential (mortality) is a dominant genetic trait in hybrid cells. Other hybrid cell lines have been isolated and are being characterized for doubling potential, karyotype and tumorigenicity in athymic nude mice.

PROJECT DESCRIPTION

Names, Titles Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

Edward Gabrielson	Medical Staff Fellow	LHC	NCI
Curtis C. Harris	Chief	LHC	NCI

Objectives:

Somatic cell genetics of mortality, tumorigenicity and other aspects of transformation will be studied using hybrids of human lung carcinoma cell lines with normal human bronchial epithelial cells.

Methods Employed:

The methods and media for culturing normal human bronchial epithelial cells have been previously developed in this laboratory. Clones of ouabain-resistant, HGPRT-lacking cells from established human lung carcinoma cell lines have been derived for the purpose of selecting hybrids.

Cell-cell fusion is done with polyethylene glycol, and hybrids are selected in a media containing HAT (hypoxanthine, aminopterin and thymidine) and ouabain. This selection media is toxic to both the normal parent (ouabain) and the carcinoma parent (HAT).

Methods for measuring the doubling potential of cell lines have been developed utilizing successive passaging of cells and colony size measurement with the Artec image analyzer. Methods for karyotypic analysis of hybrid cell lines are available and tumorigenicity may be assessed by growth in athymic nude mice.

Major Findings:

Initial fusions of HUT 292, a human lung carcinoma cell line, with normal human bronchial epithelial cells and selection of hybrids as described above has resulted in the isolation of several clones. With extended culturing, all of these clones to date have demonstrated a limited doubling potential.

Of the 24 hybrid clones from fusion of H292 DM with TBE-1 cells, 11 senesced within 20 population doublings. The 13 clones that have not senesced (and presumably are immortal) are currently being tested for tumorigenicity in nude mice.

None of the 10 hybrid clones from fusion of H292 DM with A1146 have senesced. These clones are also currently being examined for tumorigenicity.

Significance to Biomedical Research and the Program of the Institute:

Carcinogenesis appears to be a multi-step process involving multiple phenotypic changes that eventually result in malignancy. Some of these changes are cellular immortality and tumorigenicity. Study of somatic cell hybrids will hopefully lead to a better understanding of these changes and recognition of genetic regulation of the malignant phenotype.

Proposed Course:

Hybrids will be cloned and characterized for (1) population doubling potential, (2) chromosomal karyotype; and (3) tumorigenicity in athymic nuce mice.

Publications

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05325-03 LHC

PERIOD COVERED
October 1, 1984 to September 30, 1985

TITLE OF PROJECT (*80 characters or less. Title must fit on one line between the borders.*)
DNA Cytosine Methylation and Cellular Physiology

PRINCIPAL INVESTIGATOR (*List other professional personnel below the Principal Investigator.*) (*Name, title, laboratory, and institute affiliation*)

PI:	Vincent L. Wilson	Senior Staff Fellow	LHC	NCI
Others:	Curtis C. Harris	Chief	LHC	NCI
	George H. Yoakum	Senior Staff Fellow	LHC	NCI

COOPERATING UNITS (*if any*)
Clinical Hematology Branch, NHLBI, NIH, Bethesda, MD (R. J. Ley);
Gerontology Research Center, NIA, Baltimore, MD (R. G. Cutler)

LAB/BRANCH
Laboratory of Human Carcinogenesis

SECTION
Biochemical Epidemiology Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.7	0.7	1.0

CHECK APPROPRIATE BOX(ES)
☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (*Use standard unreduced type. Do not exceed the space provided.*)

The role DNA methylation plays in the promoter regions of two separate gene systems has been clarified. Demethylation of at least one Hpa II restriction site upstream from the structural coding sequences of human gamma globin or the hepatitis B core antigen gene is necessary but not sufficient for the initiation of transcription. The final conversion of a quiescent, demethylated gene (gamma globin or hepatitis B core antigen) to an active state requires some endogenous or exogenous inducing agent, which may be highly specific for any given gene or gene complex. Clonal selection is not responsible for observed changes in gene expression, since we have clearly shown that cells remethylate DNA that has been demethylated by 5-azacytidine treatment.

New micro techniques have been developed which enable the analytical quantitation of 5-methylcytosine in less than one microgram of DNA isolated from any source. Thus, the genomic 5-methylcytosine content of normal human bronchial epithelial and pulmonary mesothelial cells has been measured for the first time. These techniques have also provided for the demonstration that chemical carcinogens can induce decreases in DNA 5-methylcytosine levels in dividing normal human bronchial epithelial cells.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on This Project:

Vincent L. Wilson	Senior Staff Fellow	LHC	NCI
Curtis C. Harris	Chief	LHC	NCI
George H. Yoakum	Senior Staff Fellow	LHC	NCI

Objectives:

To determine the relationship between the changes in 5-methylcytosine patterns in DNA and the carcinogenesis process. It is known that tumor cells contain altered methylation patterns in some genes and DNA sequences as compared to normal tissue. It is not known, however, if these changes in 5-methycytosine patterns initiate carcinogenesis, occur during carcinogenesis, or are the result of this multistep process. Studies are being directed to determine the ability of chemical-carcinogens to inhibit the formation of 5-methylcytosine. These susceptible DNA sequences may be the same areas observed to be undermethylated in tumor cells. The methylation patterns of human tumor DNAs will be probed in the search for de-methylated genes and/or DNA sequences that may be specific for the tumor type or tissue of origin.

Methods Employed:

This laboratory has developed and utilized human bronchial tissue and epithelial cell culture as model for carcinogenesis studies. This system also provides a model for the study of the effects of chemical carcinogens on the methylation patterns in the DNA of normal human epithelial cells. DNAs are isolated from carcinogen-treated epithelial cultures; restricted with Hpa II, Msp I, and other enzymes sensitive to cytosine modifiction; and probed with specific DNA sequences and genes. The genomic levels of 5-methylcytosine will also be monitored in treated cells by a sensitive ^{32}P post-labeling technique developed in this laboratory. The time course of these effects will also be followed, since previous work has determined that the genomic level of 5-methylcytosine in some mammalian cells is decreased maximally by 48 hours post carcinogenic treatment. Epithelial cell DNA methylation patterns will be compared to those of various carcinoma cell lines and human tumors. High molecular weight DNAs will be isolated from human tumors; subjected to the same enzyme restriction, gel electrophoresis, as above; and probed for alterations in methylation patterns in specific genes and DNA sequences.

Major Findings:

Recent findings have determined not only that methylation patterns in DNA are important to gene expression, but also that changes in these patterns take place during differentiation and in vitro senescence. Thus, the ability of chemical carcinogens to alter 5-methylcytosine patterns in DNA may provide clues to the carcinogenic action of these agents. Previous studies have determined that the alkylation of DNA by alkylating carcinogens inhibits the enzymatic modifications of cytosine residues. Some aromatic hydrocarbon carcinogens also initiated decreases in genomic 5-methylcytosine levels in BALB/3T3 cells.

Previously, the determination of genomic 5-methylcytosine levels required the labeling of DNA in dividing cells with 6-^{3}H-uridine. Limitations in epithelial cell numbers required toxic levels of tritium in order to sufficiently label the DNA for 5-methylcytosine measurements. We have now developed a new method which is both sensitive and does not require active DNA synthesis and cell division. DNA from any source can be enzymatically digested to nucleotides and labeled with ^{32}P. The labeled nucleotides are then separated by TLC and the ratio of 5-methylcytidine to the total cytidine and 5-methylcytidine determined. This highly sensitive ^{32}P post-labeling method not only enables the above-described chemical carcinogenesis studies to be performed on human epithelial cells but also allows for monitoring of genomic 5-methylcytosine levels in tumors, tissues, and cell types from human and animal sources. Thus, changes in 5-methylcytosine levels during differentiation and during the normal aging process in vivo can now be followed.

Two separate studies have suggested that only one or two Hpa II methylation sites are important to the expression of selective genes. The human gamma globin gene in mouse erythroleukemia cells containing the human chromosome 11 was found to require the conversion of a few 5-methylcytosines to unmethylated cytosine residues at Hph II sites in the 5' leading sequences in order to be in an "allowable" state for gene expression. Subsequent treatment of the hypomethylated cells with hexamethylene bisacetamide (HMBA) was required, however, to induce active gamma globin expression in these cells. HMBA is a known inducer of globin synthesis and differentiation of erythroid cells and has been shown to alter the configuration of chromatin. Thus, the methylation pattern may be the first level of regulation of gene expression. The conversion of the quiescent gene to an active state may require demethylation followed by some endogenous or exogenous inducing agents.

This has been further supported by the finding that the expression of the transfected HBV core antigen gene in the carcinoma cell line required both the loss of methylation and subsequent cell divisions in a proper medium. The components in the medium necessary for core antigen expression are not yet known.

Significance to Biomedical Research and the Program of the Institute:

The formation and maintenance of 5-methylcytosine in mammalian DNA appears to be a dynamic system, changing during the basic biological processes of embryogenesis, differentiation, and possibly aging. Determination of what role 5-methylcytosine plays in initiating and/or controlling these biological processes would be valuable to the understanding of basic genetic mechanisms and controls in mammalian cells. Since perturbation of DNA methylation patterns may be involved in initiation and progression of carcinogenesis, advances in the understanding of the nature and functions of 5-methylcytosine should provide clues to the genetic mechanisms underlying this multistep process.

Proposed Course:

With the development of these microtechniques, there are several areas which now may be approached. A basic understanding of the role 5-methylcytosine plays in normal biological processes, including differentiation and aging, would be valuable to understanding the relationship of DNA cytosine methylation to carcinogenesis. Thus, we will determine if genomic 5-methylcytosine levels decrease during aging in vivo using selective young and old mouse tissues.

Differentiation and 5-methylcytosine will be studied using the normal human bronchial epithelial culture model system. Since chemical carcinogens induce decreases in DNA methylation levels in normal human bronchial epithelial cells, this area will be studied further using other DNA damaging agents. The progression of transfected normal bronchial epithelial cells from the phenotypically altered state to the tumorigenic state will be monitored for changes in genomic methylation levels. Depending on these results, further experiments will be performed with these cells, other model systems, such as carcinogen-treated guinea pig cells, and a chronic myelocytic leukemia animal model.

Publications

Kobra, B. E., Wilson, V. L. and Yoakum, G. H.: Induction of hepatitis B virus core gene in human cells by cytosine demethylation in the promoter. Science 220: 1103-1106, 1985.

Ley, T. J., Chiang, Y. L., Haidaris, D., Anagnow, N. P., Wilson, V. L. and Anderson, W. F.: DNA mythylation and regulation of the human β-globin-like genes in mouse erythroleukemia cells containing human chromosome 11. Proc. Natl. Acad. Sci. USA 81: 6618-6624, 1984.

Wilson, V. L. and Jones, P. A.: Chemical carcinogen-mediated decreases in DNA 5-methylsytosine content of BALB/3T3 cells. Carcinogenesis 5: 1027-1031, 1984.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05326-03 LHC

PERIOD COVERED
October 1, 1984 to Sepetember 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
HLA Antigens: Structure, Function and Disease Association

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Dean L. Mann	Medical Officer	LHC	NCI
Others:	William Blattner	Chief, Family Studies Section	EEB	NCI
	Marvin Reitz	Medical Officer	LTCB	NCI

COOPERATING UNITS *(if any)*
Laboratory of Microbiology and Immunology, NIDR (J. Oppenheim); Uniformed Services, University of the Health Sciences, Bethesda, MD (M. Newman)

LAB/BRANCH
Laboratory of Human Carcinogenesis

SECTION
Biochemical Epidemiology Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.0	0.5	0.5

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

HLA typing was performed on lymphocytes from patients with a common disease or family where one or more individuals show a common disease type. One hundred and thirty individuals with mycosis fungoides were HLA typed. A significant increase in the HLA-B35 antigen as well as HLA-DR5 was found in effected individuals compared to the normal population. HLA typing of 7 multiplex familes with members having melanoma or premalignant mole syndrome showed no linkage with the major histocompatibility complex (MHC). These data contradict earlier reports suggesting MHC association with this disease. Families with adult T-cell leukemia and HTLV-I infection (serum antibodies) were found to have a low level of linkage to MHC (code score 1.5). Patients with acquired immunodeficiency syndrome (AIDS) showed an increased frequency of HLA-DR5, MT2 in the patients with Kaposi's sarcoma. Individuals at risk for this disease (male homosexuals) who have antibodies to HTLV-III, had an increase in HLA-DR-4 compared to the normal frequency of this antigen and to the frequency of DR4 in AIDS patients. Regulation of HLA-DR expression was studied in context with HTLV-I infection. Resting ATL (adult T-cell leukemia) cells expressed little or no HLA-DR. Activation of the HTLV-I retrovirus caused a complementary increase in HLA-DR mRNA, while DNA methylation remained the same. The results indicate that post-transcriptional control of HLA-DR expression may be influenced by viral replication.

PHS 6040 (Rev. 1/84)

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

Dean L. Mann	Medical Officer	LHC	NCI
William Blattner	Chief, Family Studies Section	EEB	NCI
Marvin Reitz	Medical Officer	LTCB	NCI

Objectives:

To determine function, structure and disease association of major histocompatibility complex genes and/or their products. The studies are directed at the elucidation of genetic associations and potential genetic control of the immune response as it relates to disease process and etiology. Once markers are identified in the disease population, functional and biochemical studies are being performed in order to clearly define the genetic regulation of disease processes as it relates to immunologic response.

Methods Employed:

Standard HLA typing was performed using microcytotoxicity techniques. The technique for HLA-A,B,C has been described by Amos and Poole. The method for typing of the B lymphocytes for HLA-DR determinants was originally described by Mann et al. A total of 19 determinants controlled by the HLA-A locus, 26 alloantigens at the B locus, 6 alloantigens at the C locus, 10 alloantigens at the DR locus and 6 MT antigens were tested for in the population study. HLA typing was performed by the Laboratory of Immunology, Department of Surgery, Uniformed Services University for the Health Sciences, under an interagency agreement. The association of HLA types with disease was examined for significance by statistical methods. HLA-A,B and DR cDNA probes were provided as gifts from several U.S. and European laboratories. DNA and RNA were prepared from cell lines or peripheral blood lymphocytes. The DNA was digested with appropriate restriction in the nucleases, electrophoresed, and probed with B32 nick-translated HLA probes. Cytotoxic T cells were generated by exposure to autologous HTLV-infected cell lines. The T cells were cloned and tested for cytotoxicity to HTLV-infected cell lines.

Major Findings:

This project continues to provide significant information relevant to histocompatibility antigens that are expressed and their genetic control in the relationship to diseases. Much of the HLA typing that has been done on this project over the last year has related to HTLV studies. HLA typing has been used to monitor infections with HTLV in a variety of recipient cell lines. This typing provides not only useful information on expression of unusual HLA antigens in the infected cell lines but also the capability of monitoring the particular cell that is growing in these cultures.

The frequency of HLA antigenic determinants was studied in patients with mycosis fungoides. This is a malignant disease which has been associated with some cases of HTLV-I infections. A population of these patients from the U.S. and a population from Denmark were studied. In the latter group 18% had antibody to HTLV-I and HTLV-II retroviruses. Cells from the individuals with HTLV-I antibodies had HLA alloantigen frequencies which differed from the now infected individual. HLA-B35 and DR5 was increased (compared to normal) in frequency in both the U.S. patients and the HTLV-noninfected individuals.

Seven multiplex families with multiple cases of melanoma and premalignant mole syndrome were studied for HLA phenotypes and haplotypes. In contrast to data published by other investigaters, no linkage of this disease in the malignant and/or premalignant state with the major histocompatibility complex was found.

HLA typing of families was performed where at least one individual had adult T-cell leukemia (ATL). All family members were tested for HTLV-I infection by serologic analysis for antibodies to this virus. No specific association of an HLA haplotype was found in diseased individuals. A limited degree of association with the MHC was observed in the HTLV-I-infected population (Lode score 1.5). Out of the 12 potential haplotypes 3 had HLA-A1. No individual with this haplotype had antibody to HTLV-I. In prior studies we had demonstrated that the HLA-A1 restricted the cytotoxic activity of lymphocytes for HTLV-I infected cells. These cytotoxic T lymphocytes were obtained from a patient who had a protracted disease course which is unusual (probably rare) in HTLV-I related diseases. These observations suggest a possible mechanism for genetic control of infection and diseases. The HLA-A1 phenotype may result in development of cytotoxic T cells to HTLV-I-infected cells and thus eliminate the virus infection by the cell where the virus replicates. The 4D12 antigen (HLA-class I shared epitope) is induced in expression with viral replication in adult T-cell leukemia or with transfection experiments. The presence of this epitope as normal cells may allow viral infection in that this determinant is recognized as self. Therefore, infection may occur in those individuals exposed to the virus. Tumorigenesis may occur only in those individuals who lack HLA-A1 or some other restricting genetic factor. HLA typing of patients with AIDS and the at-risk male homosexual population demonstrated differences which may suggest that this disease has a genetic basis as well as the well-known immunologic paralysis. The at-risk population had a significant increase in HLA DR-4 compared to AIDS patients and a normal panel. HLA-DR-5 MT-2 was increased in frequency in the AIDS population compared to normals and the at-risk group. (This was frequently found in the patients with Kaposi's sarcoma. Regulation of HLA-DR expression of studied HTLV-I-infected cell lines established from patients with ATL, co-cultured cord blood lymphocytes and short-term cultures of ATL cells.) In the long-term cell culture, hypomethylation of the HLA-DR genes was found in contrast to a high degree of methylation of these genes in the ATL patients. The HTLV-I provirus is also methylated in the resting ATL cells. In 24 to 48 hour cultures of the ATL, virus is expressed as well as the HLA-DR cell surface antigens. Methylation of HLA-DR DNA remains the same during this time period, however, there is a

marked increase in mRNA. A portion of the retroviral genome is thought to have the ability to activate genes and is therefore responsible for transactivation of the provirus. Another gene responding to this portion of the provirus may be HLA-DR, since these are expressed concomitant with viral replication. Our results suggest that this control may be post-transcriptional in HTLV-I-infected cells.

Significance to Biomedical Research and the Program of the Institute:

A variety of diseases have been demonstrated to be associated with the human major histocompatibility complex. These associations and their influence in the disease process are not well understood. Further defining HLA association with disease severity demonstrates that the associations are complex and that the disease entity in and of itself probably results from gene interaction within this major histocompatibility complex. Furthermore, the demonstration that the human immune system can recognize small alterations in amino acid composition of a potential immunogen demonstrates the exquisite sensitivity of the human immune response. Alterations in immune response are well documented in patients with cancers. Whether these alterations occur as a result of or as part of the disease process and disease susceptibility remains to be determined. However, detailed analysis of the genes and gene products within the major histocompatibility complex will further our knowledge as to the role of these genes in the human immune response system and in responses that may be related to carcinogenesis.

Proposed Course:

This project represents the combined efforts of a number of investigators, both clinical and laboratory. The project is now being directed at families in which multiple cases of disease, particularly cancer, appear in a single family. Most diseases that appear to have an association with HLA have this association with the HLA-DR region of the major histocompatibility complex. Recent studies have demonstrated that this is a complex region involving at least three loci. Our work will begin to focus on the molecular heterogeneity in this region in cancer-prone families to determine particular gene structure as well as expression, and to investigate the possibility of altered HLA antigens in cancer patients. Attempts will be made to correlate combination of genes within the major histocompatibility complex with the disease. We will continue to examine alterations in immune response in in vitro assays in an attempt to correlate susceptibility and disease risk with altered immune responsiveness.

Publications

Clarke, M. F., Mann, D. L., Murray, C. and Reitz, M. S.: Differential methylation of Class I histocompatibility antigen genes in T-cell lines derived from two different types of T-cell malignancies. Leuk. Res. 8: 965, 1985.

Reitz, M. S., Mann, D. L., Trainor, C. D., Eiden, M. and Clarke, M. F.: DNA methylation and expression of HLA-DR alpha. Mol. Cell. Biol. 4: 890-897, 1984.

Reitz, M. S., Clarke, M. F., Mann, D. L., and Gallo, R. C.: Human T-cell leukemia lymphoma virus and Class I major histocompatibility antigens. In: Essox, M., Gross, L., Gallo, R. C., Human T-cell Leukemia/Lymphomas Viruses. New York, Cold Spring Harbor Laboratory. 1984, pp 181-187.

Sztein, M., Steeg, P., Oppenheim, P., Steihm, R., Mann, D. L., and Beaese, M: Modulation of human and blood monocytes DR antigen expression in vitro by lymphokines and interferon. In: Cohen, F., Oppenhiem, J. J., Interleukins, Lymphokines and Cytokines. New York, Academic Press, 1984, pp. 229-305.

Steihm, E. R., Sztein, M. B., Steeg, P. S., Mann, D. L., Newland, C., Blaese, M., and Oppenheim, J. F.: Deficient DR antigen expression on human and blood monocytes: Reversal with lymphokines. Clin Immunol. Immunopathol. 30: 430-436, 1984.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05327-03 LHC

PERIOD COVERED

October 1, 1984 to Sepetember 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Development and Use of Antibodies to Detect Carcinogen-DNA Adducts

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Dean L. Mann	Section Chief	LHC	NCI
Others:	Curtis C. Harris	Chief	LHC	NCI
	Glennwood E. Trivers	Biologist	LHC	NCI

COOPERATING UNITS *(if any)*

Department of Pathology, Uniformed Services University for the Health Sciences, Bethesda, MD (M. Newman)

LAB/BRANCH

Laboratory of Human Carcinogenesis

SECTION

Biochemical Epidemiology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.0	1.0	1.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The use of antibodies in sensitive immunoassays provides a means of detecting carcinogens adducted to DNA. In population studies, monoclonal antibody technology is a useful tool in developing reagents to detect these chemical structures. Using different monoclonal antibodies that detect different epitopes of aflatoxin adducted to DNA, patterns of reactions appear to detect different metobolic products of this carcinogen. Hetero-antibodies for benzo(a)pyrene (BP)-DNA adducts have been used to identify BP adducted to DNA in lymphocytes from individuals who by occupation have been exposed to high levels of these compounds. Serum from these individuals have been found to have antibodies to BP-DNA. Antibodies to BP-DNA have been found in these individuals as well as in individuals with no history of high exposure by occupation. Highest levels were found in some of the high exposure population although there was no correlation among the group indicating different immunologic response to this carcinogen. Antibodies to aflatoxin have also been found in some sera from individuals in high-exposure area (China), as well as from individuals in low-exposure environments (USA). Differences in titers appear to correlate with exposure areas; however, different titers suggest individual variations in these populations. Monoclonal antibodies to tobacco smoke condensate adduct to DNA have been developed.

PHS 6040 (Rev. 1/84)

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

Dean L. Mann	Section Chief	LHC	NCI
Curtis C. Harris	Chief	LHC	NCI
Glennwood E. Trivers	Biologist	LHC	NCI

Objectives:

The objectives of this project are to produce monoclonal antibodies which would react against chemical compounds known to be associated with or to be carcinogenic in animal model systems. The chemicals under study are those found in the environment and includes drugs that are known to produce cancer in man. Sera from populations exposed to these chemical carcinogens are being studied for the presence of antibodies to environmental carcinogens. These studies are epidemiologically based, including individuals from known high- and low-exposure areas.

Methods Employed:

Standard hybridoma technology is being used in this study with variations and innovations developed in this laboratory to produce human monoclonal antibodies. In the mouse system, the carcinogen-DNA adducts are combined with protamine or other adjuvants and injected into mice. After repeated immunizations, the sera from the mice are screened for antibodies to the immunogen. Once antibodies develop, the spleens are removed and fused to a HAT-sensitive mouse myeloma cell line. The established fusion product is cloned by limiting dilution and, after an appropriate period of growth, culture supernatants are tested for antibodies to the immunogen. Clones are selected for specific reactivity, expanded in tissue culture systems, and injected into the peritoneal cavity of mice in order to produce ascites fluid and high titered antibody. Analogous techniques are used in the human system. The variation that has been applied in this laboratory is to isolate peripheral blood B lymphocytes and to use these lymphocytes as fusion partners to produce monoclonal antibodies. The assay systems for antibody production are the ELISA and USERIA techniques. These techniques employ an enzyme conjugated anti-immunoglobulin directed against the monoclonal antibody which in turn detects the antigen under study. Appropriate dilutions of the monoclonal antibody are made, and the conjugated anti-immunoglobulin and substrate are added as a means of detection. The USERIA assay employs a radioactive substrate to increase the sensitivity of the methods of detection.

Human sera were tested for antibodies to carcinogen-DNA adducts using the above techniques. Sera are screened using DNA and DNA adducted with the specific carcinogen in the solid phase. After determining binding patterns, selected dilutions are tested in competitive assays to determine specificity of antibody binding. Antibodies detecting specific cell surface antigens are assayed using

the fluorescence-activated cell sorter. The monoclonal antibody is exposed to the cell, the excess removed by washing, and a fluorescein-conjugated anti-immunoglobulin is added. All cells showing a forward light scatter pattern are examined for fluorescence.

Major Findings:

Heterosera detecting benzo(a)pyrene (BP)-DNA adducts were used to assay for this adduct in DNA from lymphocytes from individuals with high occupational exposure, laboratory workers and vegetarians. BP-adducted DNA was found in individuals from each group with a higher frequency in those with occupational exposure. Using a competitive assay, sera from these individuals were tested for antibodies to BP-DNA. Some individuals from each group were positive.

Sera from individuals exposed to high levels of BP in their occupations or environment were studied for antibodies to BP-DNA adduct. High antibody titers were observed in some individuals. These titers were greater than that found in individuals not exposed by virtue of their occupation. However, there were individual variations among the high-exposure groups suggesting a variation in the immune response to these substances. Individuals with a variety of smoking-related cancers were studied for antibodies to BP-DNA. Highest frequencies were found in patients with carcinoma of the lung. Antibodies were also found in sera from individuals without disease. Differences were observed in frequency of antibody or titer comparing smokers to nonsmokers.

Human sera have also been tested for antibodies to aflatoxin (AFB). These sera were obtained from individuals chosen from areas where food contained high levels of this compound and sera from individuals from the USA where levels of AFB are low. Nearly every individual tested had antibodies to AFB. Higher titers were found in individuals in the high-exposure areas although, as with BP, individual variations suggest differences in the immune response.

Significance to Biomedical Research and the Program of the Institute:

The use of monoclonal antibody production technology is a powerful tool in biochemical epidemiology. The production of antibodies specific for chemicals, drugs or their metabolites that are associated with carcinogen can be applied to studies in which populations are screened for exposure to these environmental carcinogens. In addition, these monoclonal antibodies can be used to attempt to define specific compounds in complex materials known to be associated with carcinogenesis. Antibodies with individual specificites to a particular compound can be used to isolate the compounds and to identify specific chemicals which may be adducted to DNA. In addition, these antibodies can be used to isolate segments of DNA which have specific carcinogen complexes in order to determine the potential alteration in gene expression in cells exposed to chemical carcinogens.

Proposed Course:

The project will continue to attempt to develop a variety of monoclonal antibodies against carcinogen-DNA adducts. In turn, these antibodies will be used to study DNA from populations known to be exposed to these potential carcinogens in the environment either in the workplace or in the general population. With the appropriate techniques, sera from patients exposed to these potential chemical substances (carcinogens) will be assessed for presence of antibodies to these compounds produced by the host. Systematic screening of exposed populations, as well as control populations, will be performed. The number of chemicals will be expanded as test antigens to look for antibody in human sera in immunologic studies of cancer and control groups.

Publications

Harris, C. C., Vahakangas, K., Autrup, H., Trivers, G. E., Shamsuddin, A. K. M., Trump, B. F., Boman, B. M. and Mann, D. L.: Biochemical and molecular epidemiology of human cancer risk. In Scarpelli, D. and Craighead, J. (Eds.): The Pathologist and the Environment. New York, Williams and Wilkins (In Press)

Harris, C. C., Vahakangas, K., Newman, M., Trivers, G. E,. Mann, D. L., and Wright, W.: Detection of benzo(a)pyrene diol epoxide-DNA adducts in peripheral blood lymphocytes and antibodies to the adducts in sera from coke oven workers. Proc. Natl. Acad. Sci. (In Press)

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05328-03 LHC

PERIOD COVERED

October 1, 1984 to Sepetember 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Immunologic Studies of Human T-Cell Lymphoma Virus

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

	Name	Title	Lab	Institute
PI:	Dean L. Mann	Section Chief	LHC	NCI
Others:	Mika Popovic	Medical Officer	LTCB	NCI
	Marvin Reitz	Medical Officer	LTCB	NCI
	Robert Gallo	Chief	LTCB	NCI
	William Blattner	Chief, Family Studies Section	EEB	NCI
	Jeffrey Clark	Senior Staff Fellow	EEB	NCI

COOPERATING UNITS *(if any)*

Uniformed Services, University of the Health Sciences, Bethesda, MD (M. Newman)

LAB/BRANCH

Laboratory of Human Carcinogenesis

SECTION

Biochemical Epidemiology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.0	1.0	

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The human T-cell lymphoma virus, HTLV-I, has been found to be associated with patients with adult T-cell leukemia. Studies are underway to understand the mechanism of malignant transformation and immunologic response of individuals infected with this virus. An HLA class I epitope is expressed when cells are infected with HTLV-I but not HTLV-II. Differences in the env region of the provirus suggest that this epitope is present in the large envelope protein of HTLV-I. HTLV-I virus has been found in B-cell lines that grow spontaneously from patients with adult T-cell leukemia. The virus is expressed and infects T-cells indicating selective tropism for T-cells and not B-cells. These B-cell lines spontaneously produce acid labile and acid stable β interferon and B-cell growth factor. T-cell clones with specific function lose their specificity when infected with HTLV-I. Immunoglobulin from B-cells from chronic lymphocytic leukemia cells from patients with serum antibodies to HTLV-I have antibody activity to HTLV-I glycoproteins suggesting an indirect effect of the virus in diseases other than adult T-cell leukemia. Antibodies were raised to HTLV-I protein produced by transfection of mouse cells with a vector containing selected portions of two retroviruses. This antibody reacts with the small envelope protein (p21) and p42 the product of the pX region of the virus.

PHS 6040 (Rev. 1/84)

PROJECT DESCRIPTION

Names Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

Deal L. Mann	Section Chief	LHC	NCI
Mika Popovic	Medical Officer	LTCB	NCI
Marvin Reitz	Medical Officer	LTCB	NCI
Robert Gallo	Chief	LTCB	NCI
William Blattner	Chief, Family Studies Section	EEB	NCI
Jeffrey Clark	Senior Staff Fellow	EEB	NCI

Objectives:

To study the cell surface phenotypes, both HLA and mature lymphocyte markers, in cell lines established from patients with the human T-cell lymphoma viruses (HTLV) I and II. The study is designed to determine whether certain subpopulations of lymphocytes are infected with the virus and to examine the effect of viral infection in the expression of cell surface antigens and to characterize the viral proteins encoded for by the env region of the retrovirus by development of monoclonal antibodies. Cell surface markers were examined using the fluorescence-activated cell sorter and monoclonal antibodies directed against cell surface determinants that define subpopulations and/or specific functional subsets of lymphocytes. Indirect immunofluorescence was employed in these studies. Cell lines were established from patients with human T-cell lymphoma virus infection and/or those individuals with the associated adult T-cell leukemia. The cultures were established using PHA and T-cell growth factor and maintained in the presence of T-cell growth factor. The exception that was observed was the spontaneous growth of the B-cell lines, which were demonstrated to be infected with HTLV-I. Production of lymphokines by the HTLV-I-infected B-cell lines was studied using methodologies to assess the presence of α, β, γ interferon and B-cell growth factors. Both HTLV-I and HTLV-II were transferred into recipient cells by coculture techniques. RNA and DNA were prepared from cells by the standard methodologies. DNA and RNA were probed with the HTLV-I nick-translated probes. A shuttle vector containing SV40 and the env 3' LTR of HTLV-5 was constructed and transfected into mouse fibroblasts. Antibodies were raised to the products and monoclonal antibodies developed by hybridoma technology.

Major Findings:

Alterations in the expression of HLA alloantigens and other cell surface determinants were found in cells expressing the proviral products of HTLV-I. In all cell lines infected with the virus, there is a consistent pattern of expression of surface antigens. These antigens include the epitope detected by the 4D12 monoclonal antibody, which in turn detects an epitope that is shared with some HLA allotypic antigens. HLA-DR was present in all T-cell lines infected with the HTLV-I and -II. The receptor for T-cell growth factor, TAC, was also present in all cells that had been infected with these viruses. There was one significant alteration in the cell surface antigen expression in the HTLV-II-infected

cell lines. This was the lack of expression of the antigen detected by the monoclonal antibody, 4D12. When the cord blood lymphocytes from the same individual were infected with HTLV-I or -II, only the HTLV-I-infected cells expressed this epitope. HTLV-I and -II differ in the env region of the provirus. The results suggest that viral proteins coded for in the env region bear the epitope detected by this monoclonal antibody. The question relating to HLA epitope expression was further examined. Hamster cells were fused with HTLV-I-bearing cell lines, the fusion product cloned, the cells expanded and examined for the presence of human chromosomes, and the expression of cell surface determinants coded for by the major histocompatibility complex (chromosome 6) and the antigen bearing the epitope, 4D12. The clones that were infected with HTLV expressed the 4D12 antigen. Those clones not infected with HTLV but possessing chromosome 6 expressed HLA antigens. Other clones having neither chromosome 6 nor the virus did not express either of the above antigens. Antibodies to the HTLV-I virus have been detected in sera from patients with B-cell leukemias in other areas endemic for this HTLV-I. The frequency of this antibody reaction is above that of expected rates of infection for the general population. This is true in a group of patients from Jamaica who had chronic lymphocytic leukemia. The chronic lymphocytic leukemia cells (B cells) were examined for cell surface antigens and probed for HTLV proviral DNA. The chronic lymphocytic leukemia cells were negative for proviral DNA and only expressed the HLA-DR antigens, as well as the antigens that represent the usual B-cell phenotypes. Chronic lymphocytic leukemia cells were fused with a human myeloma cell lines and the immunoglobulin produced by the CLL captured by hybridoma technology. (The immunoglobulin from B-cell and antibody activity to the gag protein p24 from HTLV-I, -II and III.) The other reacted preferentially with HTLV-I infected T-cell lines. This observation suggests the possibility of an indirect mechanism for the influence of this virus infection on the other neoplastic conditions. Cloned cytotoxic and antigen (KLH)-responsive T cells were obtained from Drs. Flomenberg and Volkman and infected with HTLV types-I and -II. The cells were assayed for specific response. Antigen restricted cytotoxicity was lost with infection with both viruses. With KLH response, the T cells lost their ability to discriminate, responding to both HLA-DR antigen-restricted and nonrestricted antigen-presenting cells. The loss of specific T-cell function suggests that T-cell tropic viruses produce an alteration of immune response that is seen in patients with this disease.

Significance to Biomedical Research and the Program of the Institute:

The isolation of a type C retrovirus and its demonstrated association with human T-cell malignancies by Dr. Gallo and his associates has been an important advance in our understanding of neoplastic disease processes. With the isolation and capability of transferring this virus to other human cells, it is now possible to study mechanisms of regulation of malignant transformation. One important feature of malignant transformation is the particular type of cell that the virus can infect as well as alterations that may occur in the expression of cell surface antigenic determinants with viral infection. The observation that altered HLA antigen expression occurs with viral infection raises the interesting possibility

that control of infection and tumorigenesis may be related to HLA antigen expression. The coincident appearance of altered HLA expression with viral replication strongly suggests viral regulation of HLA gene expression or that the virus encodes for proteins that bear HLA alloantigenic determinants. It has been documented in other studies that the human T-cell lymphoma virus can infect individuals without producing any neoplastic disease process. The alteration in expression of HLA alloantigens may be a mechanism for control of tumorigenesis in that the viral replication induces the expression of an antigen that may be recognized as self or as foreign depending on the HLA type of the individual, and thus result in immunological control of replication and the disease process. Lymphokine production of HTLV-I infected cells by both B-cell growth factor and interferon may alter the immune response by their selective activities. Alternatively, infection with HTLV-I alters the immune response by loss of normal mechanisms to recognize self MHC antigens. The demonstration that captured immunoglobulins from CLL cells have antibody activity suggests that these cells are antigenically committed prior to malignant transformation. HTLV-I may have an indirect influence on malignancies other than T-cell leukemias. Antibodies developed to selective proteins produced by shuttle vectors suggests that the small envelope protein and the p42 px protein share common antigens and thus explain the nature of the transcription of these proteins.

Proposed Course:

Studies will continue examining the cell surface phenotypes of HTLV-infected cells. We now intend to examine the immunologic response of individuals who are infected with HTLV and who have no evidence of the leukemia and compare this response with the response of individuals who have the disease that has been associated with infection by this retrovirus. These will be done both serologically and at the cellular level. Since both the infected individuals and the patients have antibodies against the retrovirus, we will examine the differences in the antibody specificity, looking particularly at the envelope proteins of the provirus. This will be accomplished by isolation of the different problems by immunoabsorbant methods using the monoclonal antibodies developed with the shuttle vectors.

Publications

Boumpas, D. T., Hooks, J. J., Popovic, M., and Mann, D. L.: Human T-cell leukemia-lymphoma virus I and/or EB virus infected B-cell lines spontaneously produce acid labile alpha interferon. J. Clin. Immunol. (In Press)

Clark, J. W., Hahn, B. H., Mann, D. L., Wong-Staal, F., Popovic, M., Richardson, E., Strong, D. M., Loften, W. S., Blattner, W. A., Gibbs, W. N., Gallo, R. C.: Molecular and immunologic analysis of an HTLV-positive CLL case from Jamaica. Cancer Res. (In Press)

Clarke, M. F., Trainor, C. D., Mann, D. L., Gallo, R. C. and Reitz, M. S.: Methylation of human T-cell leukemia virus proviral DNA and viral RNA expression in short- and long-term cultures of infected cells. Virology 135: 97-104, 1984.

De Rossi, A., Aldovini, D., Franchini, G., Mann, D. L., Gallo, R. C. and Wong-Staal, F.: Clonal selection of T lymphocytes infected by cell-free HTLV-I virus: Parameters of virus integration and expression. Virology (In Press)

Eiden, M., Newman, M., Fisher, A., Mann, D. L., Howley, P. M., and Reitz, M. S.: HTLV-I small envelope protein expressed in mouse cells using a bovine papilloma virus-derived shuttle vector. J. Mol. Cell. Biol. (In Press)

Mann, D. L., Clark, J., Clarke, M., Reitz, M., Popovic, M., Franchini, G., Trainor, C. D., Strong, D. M., Blattner, W. A. and Gallo, R. C.: Identification of the human T-cell lymphoma virus (HTLV) in B-cell lines established from patients with adult T-cell leukemia. J. Clin. Invest. 74: 56-62, 1984.

Popovic, M., Flomenberg, N., Volkman, D. J., Mann, D. L., Fauci, A. S., DuPont, B., and Gallo, R. C.: Alteration of T-cell function by infection with HTLV-I and HTLV-II. Science 226: 459-462, 1984.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP5341-03 LHC

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT (*80 characters or less. Title must fit on one line between the borders.*)

Cytopathology of Asbestos and Other Fibers on Human Lung Cells in Culture

PRINCIPAL INVESTIGATOR (*List other professional personnel below the Principal Investigator.*) (*Name, title, laboratory, and institute affiliation*)

PI:	Edward W. Gabrielson	Medical Staff Fellow	LHC	NCI
Others:	Kaija Limmainmaa	Visiting Fellow	LHC	NCI
	Curtis C. Harris	Chief	LHC	NCI

COOPERATING UNITS (*if any*)

Duke University, Department of Pharmacology, Durham, NC (G. Rosen)

LAB/BRANCH

Laboratory of Human Carcinogenesis

SECTION

In Vitro Carcinogensis Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.0	1.0	1.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK (*Use standard unreduced type. Do not exceed the space provided.*)

Our laboratory is studying the cytopathology of asbestos fibers on human bronchial epithelial cells, human mesothelial cells and human fibroblasts in culture. Electron microscopy studies have demonstrated phagocytosis of the fibers and phase-microscopy of metaphase spreads reveal frequent attachment of the fibers to the chromosomes. Investigations regarding the mechanism of asbestos carcinogenesis suggest that oxygen radicals are probably not important intermediates. Further studies have examined the cytotoxicity of synthetic fibers, i.e., glass and carbon-graphite, on human mesothelial cell cultures.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on This Project:

Edward Gabrielson	Medical Staff Fellow	LHC	NCI
Kaija Linnainmaa	Visiting Fellow	LHC	NCI
Curtis C. Harris	Chief	LHC	NCI

Objectives:

To study the carcinogenicity and cytopathology of asbestos fibers in human mesothelial and bronchial epithelial in vitro systems. These studies include the following: (1) develop defined media for replicative mesothelial cell cutlures, (2) evaluate cytotoxicity of asbestos fibers and synthetic non-mineral fibers in mesothelial and bronchial epithelial cells, (3) evaluate the effects of asbestos fibers on progression of chromosome rearrangements in mesothelial cells, (4) evaluate asbestos as a cocarcinogen for cultured bronchial epithelial cells, and (5) evaluate the role of oxygen radicals in the mode of action of asbestos-caused carcinogenesis.

Methods Employed:

Human bronchial tissues are obtained from a medical examiner and "immediate" autopsy donors. Replicative cultures of normal bronchial epithelial cells are developed from explant culture outgrowths. Upon transfer of the explants to new dishes, the outgrowth cultures are incubated in defined, serum-free medium to expand the population, then subcultured. Mesothelial cells are obtained by centrifuging pleural effusions from donors without cancer. The fluid is centrifuged, and the pelleted cells are resuspended and inoculated into 100 mm culture dishes at a ratio of one dish per 50 ml of pleural fluid. The cells are dissociated using trypsin when the cultures attain subconfluency. The cultures are further expanded and either cryopreserved or used according to experimental protocols.

Several criteria are used to establish the identity of the cells grown in culture. Markers for bronchial epithelial cells include karyology; polygonal morphology; ultrastructural identification of tight junctions, desmsomes, and tonofilaments; production of acidic and neutral mucopolysaccharides; immunostaining of keratin; blood group antigens and type IV collagen; population doubling potential; clonal growth rate; and mitogenic responsiveness to peptide growth factors and hormones. Mesothelial cells are identified by several criteria, including immunofluorescent staining with antikeratin antibodies; a variable cell morphology depending on the presence (fusiform) or absence (cobblestone) of EGF and hydrocortisone in the growth medium; histochemical staining for hyaluronic acid-mucin; the presence of long, branched microvilli; and normal human karyotype of Giemsa-band metaphases.

Fiber cytotoxicity is assessed using clonal growth dose-response assays. Sixty-millimeter dishes are inoculated at clonal density. Twenty-four hours later, the medium is replaced with medium containing increasing concentrations of fibers. After 3 days of exposure, the fiber-treated and control cultures are rinsed twice with medium, then reincubated in fiber-free medium. Ten days post inoculation, the colonies are fixed in 10% formalin and stained with 0.25% crystal violet.

Bronchial tissue is exposed to amosite asbestos by pipetting fiber suspensions on to the epithelial surface. The explants are then submerged in medium in a stationary position for 2 hours before culturing in a rocked, controlled atmosphere chamber. The culture medium is replaced with fresh medium without fibers the next day and then at 2-day intervals. The tissues are periodically examined by light and electron microscopy.

Replicative cutlures of mesothelial cells are exposed to amosite asbestos by including the fibers (2 μg/ml) in the growth medium. After 4 days of incubation, the medium is replaced with medium without fibers and at 4-day intervals thereafter. Two weeks later, the cells are trypsin dissociated and subcultured. The following day, the cultures are reexposed to amosite asbestos. Unexposed control cutlures are carried in parallel. Giemsa banding of mesothelial metaphases is conducted to monitor chromosomal rearrangements post asbestos exposure.

Intracellular oxygen radicals are measured using electron paramagnetic resonance (EPR) with the spin trap 5,5 dimethyl-1-pyroline-1-oxide (DMPO) and free radical scavengers (N-acetylcysteine, glutathione, D-a-tocopherol and superoxide dismutase) have been added to test media to test for modification of asbestos toxicity. DNA damage by free radical mechanisms is measured by the alkaline elution technique that detects single-strand breaks.

Major Findings:

Investigations into the mechanism of asbestos carcinogenesis have shown the following: (1) Amosite asbestos (100 to 1000 μg/ml) caused focal epithelial hyperplasia and atypical squamous metaplasia inhuman tracheobronchial explants. (2) Amostie fibers were shown by both scanning and high-voltage transmission electron microsocopy to penetrate cultured epithleial cells. Short fibers (< 12 u) were found in the cytoplasm of the cells within 6 hours, whereas longer fibers incompletely entered the cells. The epithelial cells did not show marked cell surface activity, and only small membrane sleeves around noncoated fibers were observed at the points of asbestos penetration. (3) To measure toxicity, asbestos (UICC samples, 0.1 to 100 μg/ml) were added to human bronchial epithelial cells that had been subcultured 24 hours previously at clonal density. When compared to glass fibers, asbestos caused a statistically significant ($p < 0.05$) decrease in cell population doubling rate. Chrysotile was apprximately 10-fold more cytotoxic than either amosite or crocidolite. A similar order of toxicity was observed when human bronchial fibroblastic cells

were used; however, these cells tolerated approximately 100-fold more fibers for the same level of cytotoxicity. Other intracytoplasmic and intranuclear asbestos fibers were seen by X-ray microanalysis in the hyperplastic lesions. (4) Conditions for replicative cultures of mesothelial cells were developed. The clonal growth rate of mesothelial cells was first determined in nine different medium formulations, and LHC-Basal was selected as the standard nutrient mixture for mesothelial cells. Experimentation assessing numerous growth factor and hormonal supplements showed that insulin, retionic acid, EGF, and hydrocortisone spared the serum requirement (to < 3%) and enhanced the clonal growth rate of the mesothelial cells to > 0.85 PD/D. (5) Keratin, hyaluronic acid-mucin, and branching microvilli, all markers for mesothelial cells, were detected on the cells grown in the optimized medium. As assessed by Giemsa banding, the cells remain chromosomally normal until senescence (35 PD in culture). (6) Asbestos fibers are 10-fold more cytotoxic for mesothelial cells than for bronchial epithelial cells. (7) Electron microscopy studies indicate that all three types of lung cells studied readily phagocytose asbestos fibers; thus the greater sensitivity of mesothelial cells to the cytotoxic effects of asbestos cannot be explained on the basis of differences in phagocytosis. Metaphase spreads of mesothelial cells previously exposed to asbestos have demonstrated a substantial number of metaphase cells with fibers in direct contact with chromosomes. (8) Asbestos-exposed mesothelial cells had a near-normal model number of chromsomes through six successive subcultures; however, chromosome rearrangements were noted. Dicentrics were found in 10% of the fourth passage metaphases; at the sixth passage, more than half of the metaphases contained dicentrics. At the ninth subculture, 80% of the metaphases had dicentric chromosomes, and the model number had increased to 77. (9) Electron paramagnetic resonance measurements of intact cells with the spin trap DMPO failed to detect any increase in oxygen radicals in mesothelial cells after exposure to amosite asbestos. Agents known to scavange free radicals such as superoxide dismutase, reduced glutathione, N-acetylcystein and D-a-tocopherol had no effect on the dose-dependent cytotoxicity of amosite fibers. Futhermore, exposure of the mesothelial cells to amosite fibers also resulted in no significant increase in the level of DNA single-strand breaks. These results all argue against the importance of free radical mechanisms in the cytopathic effects of asbestos on cultured human mesothelial cells. (10) Code 100 thin-glass fibers have been examined for their cytotoxic effects on human mesothelial cells and are similar in toxicity to amosite asbestos fibers. Carbon graphite fibers collected from workplace settings are much less toxic than are asbestos fibers.

Significance to Biomedical Research and the Program of the Institute:

Although asbestos fibers have been epidemiologically associated as a cocarcinogen for human malignancies other than mesothelioma, these fibers are considered to be complete carcinogens for mesothelial cells. In fact, no other etiologic agent other than fibrous structures, i.e., zeolites, ceramics, and occasionally,

glass, has been identified as a causative agent for pleural and peritoneal mesothelioma. Mesothelioma is a rarely encountered malignancy. However, the latency period for this disease averages 40 years, and with the marked increase in the use of asbestos during and since World War II, an epidemic of mesothelioma has been predicted for the latter part of this century.

Proposed Course:

Growth conditions for human mesothelial cells will be continually improved. Currently, the best clue to the mechanism by which asbestos transforms mesothelial cells is the rapid appearance of chromosomal aberrations. The possitivty that asbestos fibers bind directly to chromosomes and work via mechanical mechanisms will receive additional attention. Experiments will determine if asbestos fibers bind to isolated chromosome preparations and if chemical modification of the asbestos fibers changes the chromosome binding or cytotoxic properties of asbestos. Further examination of the cytopathological effects of synthetic fibers, i.e., electron microscopy and metaphase karyotyping, will be done to develop a model system for predicting the carcinogenic potential of these agents. The possibility of unique chromosomal rearrangements resulting in the production of autocrine growth factors and/or oncogenes is being evaluated in asbestos-exposed mesothelial cells.

Publications:

Lechner, J. F., Tokiwa, T., LaVeck, M., Benedict, W. F., Banks-Schlegel, S., Yeager, H., Banergee, A., and Harris, C. C.: Asbestos-associated chromosomal changes in human mesothelial cells. Proc. Natl. Acad. Sci. USA. in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05403-02 LHC

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Molecular Analysis of Gene Regulation and Proliferative Control in Human Cells

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	Brenda I. Gerwin	Research Chemist	LHC	NCI
Other:	Kaija Linnaimnea	Visiting Fellow	LHC	NCI
	Roger Redell	Guest Researcher	LHC	NCI
	Edward Gabreilson	Medical Staff Fellow	LHC	NCI
	Peter Wirth	Expert	LHC	NCI
	Snorri Thorgeirsson	Chief	LEC	NCI
	Anna Roberts	Research Chemist	LHC	NCI
	Michael Sporn	Chief	LHC	NCI

COOPERATING UNITS (if any)

Litton Bionetics, Rockville, MD (M. Valerio)

LAB/BRANCH

Laboratory of Human Carcinogenesis

SECTION

Carcinogen Macromolecular Interaction Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.0	1.0	1.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The goal of this new project is to understand the molecular controls of mitogenesis and terminal differentiation of human lung cells. Preliminary results suggest the mesothelioma cells may escape regulatory controls by producing their own mitogen. A study has been initiated to identify critical steps in the terminal differentiation of human bronchial epithelial cells.

PHS 6040 (Rev. 1/84)

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

Brenda I. Gerwin	Research Chemist	LHC	NCI
Curtis C. Harris	Chief	LHC	NCI
Kaija . Linnaimnea	Visiting Fellow	LHC	NCI
Rojer Redell	Guest Research	LHC	NCI
Edward Gabrielson	Medical Staff Fellow	LHC	NCI
Peter Wirth	Expert	LHC	NCI
Snarri Thorgeirsson	Chief	LHC	NCI
Anna Roberts	Research Chemist	LHC	NCI
Michael Sporn	Chief	LHC	NCI

Objectives:

The goal of this project is to understand, at the molecular level, regulatory interactions of nucleic acids and proteins which govern the growth potential and differentiation status of the cells under analysis. The topics of present interest are: to establish critical differences between normal human mesothelial cells and human methothelioma cell lines; and to identify the nature of the regulatory pathways in terminal differentiation of human bronchial epithelial cells.

Methods Employed:

Normal mesothelial cells, asbestos-modified mesothelial cells, and malignant mesothelioma cells are grown in tissue culture. Whole cell RNA is prepared from these cells and from fresh or frozen tissue samples and is bound to nitrocellulose membranes and tested with radioactive DNA or RNA copies of "onc" genes in order to determine whether any of these genes are differentially expressed at different stages of the oncogenic process. Effects of growth factors and inducers of differentiation are studied for differential effects on normal mesothelial cells and mesothelioma cells.

Normal human bronchial epithelial cells and malignant cell lines of bronchial epithelial origin will be compared in the absence and presence of inducers of differentiation by 2D gel electrophoresis and by subtraction cloning in an attempt to identify critical changes which occur in normal cells in the initial phase of differentiation.

Major Findings:

It has been determined that TGFβ and H-ras expression, but not c-myc, erb-B, N-myc, or raf, is elevated in one mesothelioma line tested as compared to a number of normal mesothelial cell cultures. Furthermore, TGF appears to be a mitogen for mesothelial cells. The mesothelioma cell line has been shown to produce TGFβ and has TGFβ receptors of extremely high specificity. These studies have been recently initiated and have yet to yield definitive results.

Significance to Biomedical Research and Program of the Institute:

Mesothelioma is a human tumor known to be induced after chronic exposure by asbestos fibers. Identification of molecular differences between mesothelioma cells, normal mesothelial cells and mesothelial cells modified by asbestos treatment in vitro might lead to specific therapeutic strategies to block or reverse tumor induction.

The ability of neoplastic cells to escape the signals for normal terminal differentiation is of critical importance in the malignant process. The current study is an attempt to define, at the molecular level, some key steps in this process. Achievement of this goal would provide insight into normal differentiation controls and define pivotal steps in the development of malignancy.

Proposed Course:

We will broaden our studies to include primary mesotheliomas, other mesothelioma cell cultures, and additional primary mesothelial cells. We will attempt to develop immunocytochemical methods for detection of TGFβ and to examine the mechanism of its mitogenic effect.

We will attempt to characterize critical genes and thereby metabolic steps in the regulation of epithelial cell differentiation. These studies will utilize 2-dimensional gel electrophoresis and nucleic acid subtration techniques.

Publications

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05408-02 LHC

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Drug Metabolism Phenotyping of Nonhuman Primates

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Vincent L. Wilson	Senior Staff Fellow	LHC	NCI
Others:	Curtis C. Harris	Chief	LHC	NCI
	Susan M. Sieber	Deputy Director	DCE	NCI

COOPERATING UNITS *(if any)*

Department of Pharmacology, St. Mary's Hospital, London, England (J. R. Idle)

LAB/BRANCH

Laboratory of Human Carcinogenesis

SECTION

Biochemical Epidemiology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.5	0.2	0.3

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Phenotyping of animal models and man for their xenobiotic metabolizing capabilities has, in recent years, been undertaken in the interests of predicting carcinogenic susceptibilities. the metabolism of exogenous agents is known to be genetically dependent, and selective in vivo xenobiotic metabolic routes appear to be accessible to evaluation by the use of nontoxic doses of certain drugs. The determination of the rate of selective enzymatic modifications of test agents may thus provide a suggestion as to how susceptible an individual may be to the oncogenic potential of carcinogens activated by similar metabolic routes. Several agents including debrisoquine, S-mephenytoin, S-carboxymethyl-L-cysteine, and sulfamethazine have been shown to be metabolized by enzymatic routes governed by separate genetic loci. Thus, these agents will be used to phenotype nonhuman primates on the basis of their abilities to metabolize these compounds. Since the primate colony from which these monkeys will be sampled has been and is presently involved in ongoing chemical carcinogenesis experiments, the results of the metabolic phenotyping can be compared to the susceptibilities of the monkeys to carcinogenesis.

PHS 6040 (Rev. 1/84)

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on This Project:

Vincent L. Wilson	Senior Staff Fellow	LHC	NCI
Curtis C. Harris	Chief	LHC	NCI
Susan M. Sieber	Deputy Director	DCE	NCI

Objectives:

To determine the relationship between the genetically dependent rates of selective enzymatic reactions and the chemical carcinogenic susceptibilities in nonhuman primates, man's phylogenetically closest relative. Previous work has demonstrated that chemical carcinogens are activated by selective enzymatic routes in vitro and in rodent animal models. Some correlation has been found between the rates of activation of some carcinogens and the carcinogenic susceptibilities of rats as well. Several studies have clearly demonstrated human polymorphisms in the rates of some of these metabolic routes. However, the correlation of these human metabolic phenotypes with cancer susceptibility has to be based solely on epidemiological data. Thus, more phenotyping data are needed from animal models phylogenetically closer to man than rodents and where carcinogenesis data are available.

Methods Employed:

Subefficacious doses of debrisoquine (DBQ), S-mephenytoin (MPH), S-carboxymethyl-L-cysteine (SCMC), or sulfamethazine will be administered p.o. to monkeys, and 24-hour urine samples collected and analyzed for parent drug and metabolites(s). The methods for quantitation of these compounds in urine have been previously reported in human studies. These four agents were chosen because they each represent a metabolic phenotype governed by separate genetic loci. The enzymatic reactions monitored by these agents are aliphatic ring hydroxylation by DBQ aromatic ring hydroxylation by MPH, S-oxidation by SCMC, and N-acetylation by sulfamethazine.

The results of phenotyping will be compared with the known carcinogenic susceptibilities of these monkeys.

Major Findings:

Preliminary results of phenotyping three monkeys from each of three species, rhesus, cynomolgus, and African green monkeys, are available. All of the monkeys tested rapidly metabolized DMQ, MPH, and SCMC. The metabolic ratio (concentration of parent drug to metabolite) was observed to range from 0.02 to 0.6 for DBQ, which is well below that reported for man. No differences between species were observed with the minimal sample size of three monkeys per group. MPH metabolism was extensive in these monkeys as well. From 6% to

52% of the administered dose was excreted as 4 hydroxy-MPH in 24 hours, which is greater than that reported for man. There were no significant differences in the rate of MPH hydroxylation between species. The rate of sulfoxidation of SCMC was also high in these monkeys. The metabolic ratio ranged between 0.8 and 3.8, but the rhesus and cynomolgus monkeys metabolized SCMC significantly ($P < 0.10$) faster than the African green monkeys. The average SCMC sulfoxidation index per species was 1.4 ± 0.3, 1.2 ± 0.3, and 2.7 ± 0.9 for rhesus, cynomolgus, and African greens, respectively. These rates are more rapid than that reported for man. Rhesus monkeys were significantly ($P < 0.01$) slower N-acetylators of sulfamethazine than African green monkeys, while the rate in the cynomolgus monkeys fell between the former two species. The average rate of N-acetyl-sulfamethazine formation was $51 \pm 4\%$, $65 \pm 12\%$, and $76 \pm 9\%$ for rhesus, cynomolgus, and African green monkeys, respectively. This rate of sulfamethazine acetylation in rhesus monkeys compares well with previously reported values. These rates also compare well with the human data, classifying the rhesus as poor N-acetylators, the African greens as extensive N-acetylators, and individual cynomolgus monkeys in both categories.

The rapid rate of enzymatic hydroxylation observed in these monkeys for DBQ and MPH would suggest that these monkeys would be susceptible to carcinogenesis upon exposure to aromatic hydrocarbon carcinogens. Aflatoxin B_1 and methylaoxy-methanol-acetate did produce tumors in these species (Adamson and Sieber in Langenbach, R., Nesnow, S. and Rice, J. M. [Eds.]: Organ and Species Specificity in Chemical Carcinogenesis, New York, Plenum Press, 1983, pp. 129-140). However, several other carcinogens, including benzo[a]pyrene, 3-methylcholanthrene, and cigarette smoke condensate, did not induce tumors. Thus, there may be some correlation between the metabolic phenotype and chemical carcinogenesis susceptibility, but the data presently available are insufficient to draw firm conclusions.

Significance to Biomedical Research and the Program of the Institute:

A strong correlation between xenobiotic metabolism phenotypes and chemical carcinogenic susceptibilities in nonhuman primates would provide a firm basis for monitoring people for selective susceptible metabolic phenotypes. Individuals found to be extensive metabolizers of agents such as DBQ and MPH may be more susceptible to aromatic hydrocarbon carcinogens. Thus, these extensive metabolizers should not smoke and should avoid jobs that provide high risk of exposure to aromatic carcinogens. Evaluating the human population for individuals who may be at high risk of cancer from chemical carcinogens may, therefore, ultimately provide a prophylactic treatment of this disease.

Proposed Course:

The preliminary results from three monkeys from each of three species did not provide any polymorphisms in phenotypes within a given species. Several monkeys from one species will be phenotyped as described above in the search for metabolic

polymorphisms. Since the suggested predictability of cancer susceptibility is based on polymorphisms within the human population, similar findings within the monkey colony would enhance these studies. Subsequent to further phenotyping of monkeys, selective monkeys will be sacrificed and organ and tissue culture studies initiated. The xenobiotic-metabolizing and carcinogen-activating capabilities of these cultures will be used to compare the in vivo phenotyping data and the site(s) of tumors in chemical carcinogenic susceptible monkeys.

Publications

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01CP05409-02 LHC

PERIOD COVERED
October 1, 1984 to September 30, 1985

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)
Control of Growth and Differentiation of Human Bronchial Epithelial Cells

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	Tohru Masui	Visiting Fellow	LHC	NIH
Others:	Curtis C. Harris	Chief	LHC	NIH
	LaLage Wakefield	Visiting Fellow	LCP	NCI
	Michael Sporn	Chief	LCP	NCI

COOPERATING UNITS (if any) Univ. of Maryland School of Medicine, Baltimore, MD (B.F. Trump); Georgetown University School of Medicine, Washington, DC (H. Yeager); VA Hospital, Washington, DC (P. Schafer); Centers for Disease Control, Atlanta, GA (J.Lechner)

LAB/BRANCH
Laboratory of Human Carcinogenesis

SECTION
In Vitro Carcinogenesis Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.2	1.2	1.0

CHECK APPROPRIATE BOX(ES)
☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Defined methods to grow replicative cultures of normal human bronchial epithelial (NHBE) cells without serum have been developed. These cells can be subcultured several times; will undergo 35 population doublings; and have expected epithelial cell characteristics of keratin, desmosomes and blood group antigens on their cell surface. NHBE cells inoculated at clonal density will multiply with an average generation time of 28 hr; the majority of the cells are small and migratory and have few tonofilaments. Adding human whole blood-derived serum (BDS) depresses the clonal growth rate of NHBE cells in a dose-dependent fashion. In contrast, 10 representative lines of human lung carcinomas either replicate poorly or fail to grow at all when inoculated at clonal density in serum-free medium. Their rates of multiplication increase in direct proportion to the amount of BDS added to the optimized medium. BDS reduces the clonal growth rate of NHBE cells by specifically inducing squamous differentiation. The differentiation-inducing activity was not present in plasma but was found in platelet lysates. TGF-beta was found to the primary differentiation-inducing factor in serum for NHBE cells, while TGF-beta was not growth inhibitory for malignant cells. These differential effects of TGF-beta on normal versus malignant cells are not because of lack of TGF-beta specific receptors on malignant cells.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on This Project:

Tohru Masui	Visiting Fellow	LHC	NIH
Curtis C. Harris	Chief	LHC	NIH

Objectives:

To develop systems to study mechanisms involved during malignant transformation of human epithelial cells. These studies include the following: (1) develop defined media for replicative epithelial cell cultures from bronchial tissues; (2) develop efficient assays to quantify the various squamous differentiation-inducing factors; (3) identify and characterize a factor in BDS that induces squamous differentiation; (4) identify and characterize an autocrine growth factor; (5) identify and characterize an autocrine squamous differentiation-inducing factor; (6) elucidate the pathways of squamous differentiation and determine abberrations that cause human lung carcinoma cells not to respond to these squamous differentiation-inducing factors; (7) elucidate the growth response of normal epithelial cells and determine aberrations that induce differences in growth factor dependencies of human lung carcinomas.

Methods Employed:

Human bronchial tissues are obtained from a medical examiner and "immediate" autopsy donors. Bronchial tissues are dissected from surrounding stroma, cut into 0.5 cm square pieces and used to establish explant cultures. Replicative cultures of normal bronchial epithelial cells are developed from explant culture outgrowths. Upon transfer of the explants to new dishes, the outgrowth cultures remaining in the original dishes are incubated in defined serum-free medium to expand the population and are then subcultured. These normal human bronchial epithelial (NHBE) cells are used in growth and differentiation studies or are cryopreserved for future use. Mitogenicity is quantified by measuring the clonal growth rate, rate of incorporation of ^{3}H-thymidine into acid precipitable material, and the labeling index by autoradiography. Squamous differentiation is determined by measuring the cell area, extra cellular plasminogen activator activity, Ca ionophore-induced cross-linked envelope formation. Since epinephrine antagonizes the effect of TGF-β , LHC-8 (LHC-9 medium without epinephrine and retinoic acid) is used mainly in the experiments.

Major Findings:

Human bronchial epithelial cell culture experiments have yielded the following results. A method for routinely initiating replicative epithelial cell cultures of human bronchus was developed. Large pieces of bronchus tissue were initially set up as explant cultures and incubated in a rocking chamber for 7-10 days to facilitate reversal of ischemia. The explants were then cut into smaller pieces, explanted and incubated in a serum-free medium

optimized for growth of NHBE cells. This medium (LHC-9) permits rapid outgrowth of epithelium but retards growth of the fibroblastic cells. The medium is a modification of MCDB 152. The changes are as follows: the concentrations of arginine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, tyrosine, valine, choline and serine are doubled; the concentrations of $MgCl_2 \cdot 6H_2O$ and $CaCl_2 \cdot 2H_2O$ are increased 3.5 times; the concentrations of NaCl and HEPES buffer are reduced 20% and the concentration of Na_2HCO_3 is reduced 15%. LHC-9 is supplemented with insulin, 8.7×10^{-7} M; epidermal growth factor (EGF), 8.25×10^{-10} M; transferrin, 1.25×10^{-7} M; phosphoethanolamine, 5×10^{-7} M; triiodothyronine, 1×10^{-9} M; epinephrine, 1.6×10^{-6} M; retinoic acid, 3×10^{-10} M; bovine pituitary extract, 35 µg protein/ml and gentamycin, 50 µg/ml.

Several markers that can be used as quantitative assays for squamous differentiation-inducing activity were determined as the following: (1) increase in cross-linked envelope formation; (2) increase in cell area; (3) increase in extracellular plasminogen activator activity; (4) decrease in clonal growth rate; (5) irreversible inhibition of ^{3}H-thymidine incorporation.

Supplementation of LHC-8 with as little as 1% fetal bovine whole blood-derived serum (BDS) resulted in a decrease in clonal growth rate; 8% supplementation completely inhibited growth by inducing terminal squamous differentiation. Human lung carcinoma lines were also incubated in LHC-8 medium with and without 8% BDS. The results showed that serum toxicity per se was not responsible for the observed inhibition of NHBE cell growth; all 10 carcinoma lines divided significantly more rapidly ($p < 0.05$) in BDS-supplemented medium. Thus, the carcinoma cells have both increased requirements for BDS mitogens and a greatly reduced ability to respond to a factor in BDS that induces the normal cells to undergo squamous differentiation. Immunoperoxidase staining for involucrin clearly revealed that NHBE cells exposed to BDS were arranged in a multilayered fashion. The overlying cells were large and strongly involucrin positive, whereas the basal cell sheets were involucrin negative.

Type β transforming growth factor (TGF-β) isolated from human platelets was studied as the serum factor responsible for inducing NHBE cells to undergo squamous differentiation. NHBE cells were shown to have high affinity receptors for TGF-β. TGF-β induced the following markers of terminal squamous differentiation in NHBE cells: (1) increase in Ca ionophore-induced formation of cross-linked envelopes; (2) increase in extracellular activity of plasminogen activator; (3) irreversible inhibition of DNA synthesis; (4) decrease in clonal growth rate; (5) increase in cell area. The IgG fraction of anti-TGF-β antiserum prevented both the inhibition of DNA synthesis and the induction of differentiation by either TGF-β or blood-derived serum. Therefore, TGF-β is the primary differentiation-inducing factor in serum for NHBE cells. TGF-β did not inhibit growth of human lung carcinoma cell lines and Ha-ras oncogene transfected NHBE cells in the serum-free monolayer culture system. TGF-β-specific receptor assay revealed that the differential effects of TGF-β on NHBE cells and human lung malignant

cells are not because of lack of TGF-β receptors on malignant cells. Epinephrine antagonized TGF-β-induced inhibition of DNA synthesis and squamous differentiation of NHBE cells. Although epinephrine increased cyclic AMP levels in NHBE cells, TGF-β did not alter the cyclic AMP levels in NHBE cells either in the presence or absence of epinephrine. Therefore, the action of epinephrine on TGF-β effect appears to be via indirect mechanisms.

Cell density was found to influence the effect of Ca^{2+} on growth. Whereas optimal growth occurred at clonal densities in medium containing 1 mM Ca^{2+}, rapid squamous terminal differentiation occurred when the medium of dividing high-density cultures was changed from 0.1 to 1 mM Ca^{2+}. These observations suggest that the Ca^{2+} concentration influences the activity of an autocrine squamous differentiation-inducing factor. This autocrine differentiation-inducing factor may be TGF-β, because TGF-β is a very potent differentiation inducer (ID_{50} for DNA synthesis is 0.4 PM in LHC-8 medium) and also has been found in various normal tissues.

There was significantly less inhibition of NHBE cell growth with plasma-derived serum. On the other hand, platelet-derived growth factor (PDGF) at concentrations that stimulated fibroblastic cell multiplication also inhibited DNA synthesis and stimulated terminal differentiation of NHBE cells. This commercially available PDGF effect on NHBE cells was due to TGF-β contamination.

An autogenous growth factor was detected by measuring growth rate as a function of cell density. This factor is found in NHBE cell conditioned medium. NHBE cell conditioned medium will increase the growth rate of NHBE cells incubated in LHC-8 medium. In addition, the conditioned medium stimulates multiplication of normal human bronchial fibroblasts and mouse lymphocytes. Interleukin 1 was found to increase the growth rate of NHBE cells. Further, interleukin 1 was detected in NHBE cells by immunoperoxidase staining. These observations suggest that the autocrine growth factor may be interleukin 1.

Significance to Biomedical Research and the Program of the Institute:

Understanding those processes that control growth and differentiation of normal human epithelial cells and elucidating how these controlling mechanisms differ in carcinoma cells are central to our understanding of carcinogenesis. Further, these differences in control processes may then be exploited both to identify premalignant cells and to design new novel chemoprevention modalities.

Proposed Course:

Since TGF-β was found to be the differentiation-inducing factor, we can use TGF-β as a probe to study differences between normal and malignant cells. The preliminary experiments showed that diacylglycerol (DAG) induces squamous differentiation of normal cells. Effects of TGF-β and DAG were neutralized by epinephrine. It is feasible that diacylglycerol could be the second messenger of signal transduction system with which the GTP binding protein system activated by epinephrine could interfere. This working hypothesis will be investigated.

Our studies indicate that the TGF-β differential effects on normal and malignant cells are not due to lack of TGF-β-specific receptors on malignant cells.

TGF-β and DAG may also play an important role in autocrine mechanisms. The autocrine growth factor will be purified using HPLC columns and characterized. In addition, the mitogenic potency and specific binding of this factor will be determined for both NHBE and tumorigenic lung cells.

Publications

Lechner, J. F.: Nutrient, hormone, growth factor and substrate interdependent regulation of epithelial cells growth. Fed. Proc. 43: 116-120, 1984.

Lechner, J. F., Haugen, A., McClendon, I. A. and Shamsuddin, A.: Induction of squamous differentiation of normal human bronchial epithelial cells by small amounts of serum. Differentiation 25: 229-237, 1984.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05410-02 LHC

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Hepatitis B Virus Carcinogenesis

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	George H. Yoakum	Senior Staff Fellow	LHC	NCI
Others:	Vincent Wilson	Senior Staff Fellow	LHC	NCI
	Dimitrios Boumpas	Visiting Fellow	LHC	NCI
	Curtis C. Harris	Chief	LHC	NCI

COOPERATING UNITS (if any)

Cancer Institute, Chinese Academy of Medical Science, Beijing, Peoples Republic of China (Sun Tsung-tang and Hsia Chu-chieh); Department of Pathology, University of Maryland, Baltimore, MD (B. F. Trump)

LAB/BRANCH

Laboratory of Human Carcinogenesis

SECTION

Carcinogen Macromolecular Interaction Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.5	1.0	0.5

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The role of hepatitis B virus (HBV) in human cancer is being investigated by i) developing recombinant human cell lines that carry and express HBV genes providing in vitro models to study the interaction between HBV and human cells; and ii) HBV risk-group patients, lymphocytes and nucleic acids are being analyzed for the presence of HBV. The development of a recombinant human epithelial cell line (GTC2) that contains only the HBV core antigen gene (HBc) provides a model system to study the regulation and expression of an HBV gene required for virus replication. The discovery that HBc gene expression is cytopathologic when expression reaches maximal levels, and the observation that 5-methylcytosine and nutritional factors regulate the expression of the HBc gene indicates that the role HBV infection plays must include the promotional effect of increased cell division following the direct destruction of liver cells. HBc gene expression in human epithelial cells is controlled by the methylation of an Hpa II site located 280 base pairs upstream from the structural gene. The detection of replicative forms of HBV in lymphocytes from chronic active hepatitis (CAH) and acquired immunodeficiency syndrome disease (AIDS), and the stimulation of HBc gene expression when GTC2 or PLC/PRF/5 cells are treated with 100 u of alpha interferon (a-IFN) suggest that HBV may have a cytolytic effect during the infection of lymphocytes that is important to the immunological abnormalities frequently associated with HBV infection.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

George H. Yoakum	Senior Staff Fellow	LHC	NCI
Vincent Wilson	Senior Staff Fellow	LHC	NCI
Dimitrios Boumpas	Visiting Fellow	LHC	NCI
Curtis C. Harris	Chief	LHC	NCI

Objectives:

The role of hepatitis B virus in human hepatocellular carcinoma is being studied in vitro by investigating direct mechansism of HBV pathology and determining the regulation and biological effects of the hepatitis B virus core antigen gene (HBc) in human cells. This research program focuses on (1) the mechanisms of HBV pathology and its role in carcinogenesis and (2) the mechanism of biological responses involved in these carcinogenic processes with potential application to cancer epidemiology and disease prevention.

Methods Employed:

We have developed a method to genetically transfect a variety of human cell types, including normal fibroblastic and epithelial cells, carcinoma cells, transformed fibroblasts, and lymphoid cell lines. Introduction of HBV and HBV genes into human cells is essential to the development of effective research programs to study the role of HBV in human carcinogenesis at the genetic and molecular levels because there is no system available at this time to infect cells with HBV in tissue culture. The protoplast fusion transfection procedure permits transient expression of transferred genes in 70-90% of the recipient cell cultures for 6-12 days after the procedure. This permits the construction of human cell lines carrying HBV for in vitro characterization of virus pathology and carcinogenic potential. The protoplast fusion method of transfection for transfer of plasmids stably transfers genes into human cells at frequencies greater than 10^{-3} units.

Standard nucleic acid hybridization analysis methods will be utilized to characterize the genetic organization of HBV transfected into human recombinant cell lines constructed for these studies. This includes slot-blot hybridization of DNA or RNA products to detect genomic DNA inserts and characterize transfected gene transcription products. Southern hybridization analysis of the genetic organization of HBV transfectants permits interpretation of gene expression experiments and testing of the methylation state of the transfected virus genome.

The survey of patient tissues for HBV DNA sequences and transcripts by slot-blot hybridization analysis and Southern hybridization requires coordination with the Family Studies Section, NCI to obtain appropriately classified human tissues from HBV-risk groups for this study. Selected samples of patient tissue DNA have been probed by Southern hybridization analysis to test for the presence of replicative HBV DNA.

Major Findings:

Progress during the previous year includes the mapping of an Hpa II site 280 base pairs upstream for the HBc structural gene (Hpa II $^{-280}$) responsible for the methylation regulation of HBc gene expression. In addition, the induction of HBV gene expression after treatment of GTC2 or PLC/PRF/5 cells with a-IFN, and the finding that both replicative and integrated HBV DNA is present in the lymphocytes of CAH and AIDS patients are important to our understanding of the mechanism of HBV carcinogenesis.

Since the transfected HBV sequences carried by recombinant human cell line GTC2 contain only the HBc gene and regulatory elements intact between two Bam H1 sites (or two flanking Ava I sites), this cell line provides a model to determine the role of site-specific methylation in HBc gene regulation. The methylation state at Hpa II^{-479} was determined by digestion of DNA from nuclei of GTC2 cells with Bam HI or Ava I to release the transfected HBc gene. The DNA was subsequently digested with Hpa II before separation of the fragments by gel electrophoresis for Southern hybridization. Evaluation of site-specific methylation in the HBc gene of GTC2 cells after treatment with 5'-azacytidine is possible because the ratio of the HBV-hybridizing DNA fragments varies directly with the methylation state at the relevant Hpa II sites. A mechanism for 5'-azacytidine induction by Hpa II^{-280} hypomethylation of the HBc gene includes the initiation of activated transcription followed by reduced the ability of the DNA cytosine methylase to maintain the methylation state at Hpa II^{-280} by exclusion.

The effect of IFN on HBV gene expression was determined by measuring HBVtranscription in GTC-2 and PLC/PRF/5 cells after treatment with 100 units of aIFN. The data from three expriements indicate that a-IFN treatment increases the level of transcription of the HBsAg and HBcAg genes.

Southern hybridization analysis of DNA from PBL and LN tissues of AIDS and CAH patients reveals the presence of replicative intermediates of HBV DNA. The gels resolved two hybridizing bands at 3.2 and 6.0 Kbp for DNA prepared from fresh LN and PBL tissues from AIDS and 1/3 of CAH patients tested. The presence of the correct size HBV DNA (3.2 Kbp) and a molecular weight species aproximately 1.9-fold the packaged size of the virus (6.0 Kbp) indicates that the PBL and LN tissues from these three patient groups are actively infected with HBV; the 1.9 Kbp observed in DNA from one CAH patient's PBL nucleic acids could be a supercoiled intermediate of HBV replication.

HBV infection of lymphocytes from patients with AIDS, and chronic active hepatitis (CAH) is detectable by nucleic acid hybridization analysis. The infection of immune system cells by HBV may be important in the pathogenesis and immunological abnormalities associated with these conditions.

Significance to Biomedical Research and the Program of the Institute:

HBV infection is a serious worldwide health problem that is pathologically linked to viral hepatitis and liver cancer. There are an estimated 200 million individuals whose serum is positive for the surface antigen, HBsAg.

The surface antigen and the core antigen (HBcAg) have different roles during HBV infection. Serologic data suggest that the constitutively regulated gene for HBsAg (HBs) is needed for expansion of the focus of viral infection because antibodies to HBsAg are required for protection against the virus by immunization and are present during recovery from infection. In contrast, antibodies to the HBV core gene (HBc) product do not appear consistently during recovery and are frequently associated with virus replication and consequent infectivity of patients sera. To understand the mechanism of HBV pathology during acute and chronic disease processes, it is essential to separate the various viral genetic elements and to study their biological effects and molecular biology in a model cell system in vitro. This project demonstrates the value of such an approach by revealing the importance of the core antigen in the cytotoxic response of cells to infection with HBV, a role previously unassigned to any specific HBV gene. The methylation state of chromosomal DNA has been implicated as a general controlling factor in carcinogenesis. The system described here provides a unique opportunity to study, at the molecular level, the role of DNA cytosine methylation in controlling the experssion of a specific gene of established biological importance in human cells.

Proposed Course:

Experiments are currently underway to transfect primary cultures of normal human hepatocytes with cloned HBV DNA in collaboration with Dr. I.-C. Hsu of the University of Maryland School of Medicine. A similar collaboration with Dr.Sun Tsung-tang of the Cancer Institute, Chinese Academy of Medical Science, has been established. In addition, Dr. Sun and his colleagues are implementing a hybridization screening of lymphocytes from patients with CAH and/or hepatocellular carcinoma for HBV DNA to determine the role of lymphocyte infection in the disease process. Future plans include a further characterization of the factors that regulate HBc gene expression and its biological consequences.

Publications:

Korba, B. E., Wilson, V. L., and Yoakum, G. H.: Induction of hepatitis B virus core gene in human cells by cytosine demethylation in the promoter. Science 228: 1103-1106, 1985.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01CP05413-02 LHC

PERIOD COVERED
October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Enzyme Immunoassay for Endogenous N-Nitrosation Products

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Glennwood E. Trivers	Biologist	LHC	NIH
Others:	Curtis C. Harris	Chief	LHC	NIH

COOPERATING UNITS *(if any)*
Department of Nutrition and Food Science, MIT, Cambridge, MA (S. Tannenbaum)

LAB/BRANCH
Laboratory of Human Carcinogenesis

SECTION
Biochemical Epidemiology Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.4	0.4	0.0

CHECK APPROPRIATE BOX(ES)
☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Endogenous nitrosation of amino acids and related molecules is an important potential source of carcinogen N-nitroso compounds in human populations. Of the several nitroso compounds (N-nitrososarcosine, N-hydroxynitrosoproline and N-nitrosopyrrolidine, etc.) shown to occur in human urine and food stuffs, NPro is one that is stable, nonmutagenic and noncarcinogenic and can be safely given to humans. Ingested preformed NPro is excreted unchanged in human and animal urine and its concentration in urine increases proportionally to increases in percursor nitrates and L-prolines consumed, respectively, in food stuffs and drinking water. In addition, higher levels of urinary NPro have been found in smokers than in nonsmokers and in individuals residing in geographical areas with high incidence of stomach and esophageal cancer than in persons from areas with low incidence of these diseases. Studies of urinary NPro are currently accomplished by a measure of in vitro methylated urinary NPro (NPro [CH_3]) in gas chromatography with a thermal energy analyzer (GC-TEA). We have developed an immunoassay for NPro (CH_3) which may simplify and facilitate the study of this potentially important phenomenon. Our rabbit and anti-KLH-NPro antisera does not recognize proline nor unconjugated NPro, but does recognize the methyl ester of NPro (NPro [CH_3]) and nitroprolylglycine (NPG). Nine esterfied extracts of human urine previously tested positive in GC-TEA have been tested positive in ELISA, and all measured apparently higher in GC-TEA. Characterization of the antibody is incomplete and the quantitative difference may be due to detection of substances other than NPro. However, NPG is not known to be present in biological materials.

PHS 6040 (Rev. 1/84)

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

Glennwood E. Trivers	Biologist	LHC	NIH
Curtis C. Harris	Chief	LHC	NIH

Objectives:

To develop simplified enzyme immunoassay (EIA) and ultrasensitive enzymatic radioimmunoassay (USERIA) procedures for detection and quantitation of N-nitrosoproline (NPro) in human and animal tissues and fluid specimens; to study NPro levels in individuals and populations potentially at risk for developing related cancer; to study NPro in environmental and experimental conditions involving known and suspected chemical carcinogenesis.

Methods Employed:

Bovine serum albumin (BSA) coupled to NPro and keyhole limpet hemanocyamin (KLH) coupled to NPro are used as immunogens in Freund's complete adjuvant. Three rabbits, each given serial injections of one or the other immunogen then bled periodically for serum, to be tested for antibody activity. Antisera to BSA and KLH (Cappel) were used respectively to verify the binding of the NPro conjugates to polyvinyl microtiter plates; BSA-NPro-coated plates used to screen sera from KLH-NPro-injected rabbits and vice versa. Active sera were tested in noncompetitive EIAs to titer antibody and subsequently in competitive EIAs for the ability of the fluid phase conjugates to compete for the antibody. Standard curves of the methyl ester of NPro (NPro [CH_3]) were performed in 2% horse serum containing either 5% or 10% methanol.

Major Findings:

Each of three rabbits given intramuscular injections of BSA-NPro and KLH-NPro developed antisera with titers greater than 1:800,000 when tested in noncompetitive assays against 0.2 ng in the solid phase for EIA. Antisera reactions in homologous systems (BSA-NPro antisera vs. BSA-NPro and vice versa) compared to reactions in heterologous systems (BSA-NPro antisera vs. KLH-NPro and vice versa) demonstrated that the BSA-NPro antisera antibodies were directed primarily (70%) against the carrier, while the KLH-NPro antiserum is 80% against the hapten. Consequently, the KLH-NPro antiserum was used to establish conditions for a competitive EIA using BSA-NPro as the solid phase immunosorbent, with virtually no background reaction with BSA. With 1:400,000 dilution of KLH-NPro antiserum, the detection minimum in USERIA with 0.2 ng as immunosorbent is 0.1 picogram (pg) of BSA-NPro, 0.02 pg NPro and 50% inhibition (50 IH) of 2.0 and 0.2 pg, respectively. Minimum detection of the BSA-NPro in competitive ELISA is established at 6.0 pg (50 IH = 20 pg). Nevertheless, the antisera does not detect uncoupled NPro in noncompetitive or competitive EIA and RIA. In addition, the KLH-NPro antiserum does not react with either D, L or DL prolines, D or L prolinamide, or N-nitroso-L-thiazolidine-4-carboxcylic acid, the new nitroso compound recently discovered in human urine. However, we have recently demonstrated competitive recognition of both NPro (CH_3) and nitrosoprolylglycine (NPG), a nonbiological substance. Apparently, the major epitopes in the conjugates

are modifications of the hapten that are the result of the conjugation method. Using NPro (CH_3) as the fluid phase competitor, conditions for biological testing have been established with 0.5 ng BSA-NPro as the immunosorbent. Minimum detection of NPro (CH_3) and NPG are 6 ng and 1 ng, respectively. Nine esterfied extracts of human urine dissolved in methanol and previously tested positive in GC-TEA have been tested positive in the ELISA. Using a final concentration of 2.5% or 5% of the sample in horse serum, quantitation was performed on a standard curve established in buffered horse serum containing 2.5% or 5% methanol. All nine samples measured apparently higher in the ELISA than in GC-TEA. Characterization of the antibody is incomplete and the quantitative difference may be due to detection of a substance(s) other than NPro. However, NPG is not known to be present in biological materials or systems.

Significance to Biomedical Research and the Program of the Institution:

A simplified, more sensitive assay for NPro will economize and make practical the assessment of endogenous NPro for clinical and research purposes. An easily assessed measure of endogenous nitrosation will be an important addition to the technology for studies of human carcinogenesis. Such studies can predictably further our understanding of the pathobiological consequences of the potentially additive or synergistic effects of measurable endogenous and exogenous exposures to chemical carcinogens in human populations. Ultimately, such studies will allow the definition of those who are "predisposed" for the development of cancer by virtue of a measurable "carcinogen burden." This important step will allow the possibly significant differentiation between cancerous and noncancerous individuals and subgroups in the populations of heavy smokers; asbestos, coke oven and aluminum workers at risk for malignancies of the stomach, esophagus, bronchus and lung; and individuals in geographically endemic areas for high incidence of hepatoma associated with aflatoxin.

Proposed Course:

The immediate course for this study will concern the completion of the antiserum characterization. Currently, the recognition of NPro (CH_3) has allowed preliminary detection of a level of NPro in human urine samples that may be correctly higher than amounts measured by GC-TEA for the same samples. To address this question we will test urinary NPro samples with and without treatment with dichloromethane, which methylates NPro and presumably produces antibody recognition and inhibition in the competitive assay; NPro(CH_3) samples with and without previous x-irradiation which dissociates the methyl group and should prevent inhibition in the competitive assay; NPro(CH_3) samples before and after complete drying (under nitrogen or argon) which destroys the methyl group and theoretically the antibody recognition. These proposed tests will determine the authenticity of the assumed antigenic nature of NPro(CH_3) and establish or eliminate the possibility of a substance(s) other than the NPro in human urine that reacts with this putative anti-NPro(CH_3) antibody. Subsequently, the EIA and a soon-to-be established USERIA for NPro(CH_3) will be used in preliminary stages to confirm its comparative applicability for NPro studies when compared with GC-TEA methodology.

Publications

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05421-01 LHC

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Genetic Transfection of Human Cells

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	George H. Yoakum	Senior Staff Fellow	LHC	NCI
Others:	Louise Malan-Shibley	Microbiologist	LHC	NCI
	Paul Amstad	Visiting Fellow	LHC	NCI
	Curtis C. Harris	Chief	LHC	NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Human Carcinogenesis

SECTION

Carcinogen Macromolecular Interaction Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.0	1.5	0.5

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Protoplast-fusion allows efficient, stable transfection of genes carried on plasmid vectors into human cells grown in serum-free media. It provides a new tool for genetic analysis of human cells in vitro, and the means to construct specialized human cells expressing gene products of economic importance. This gene-transfer method originated as a means to fuse dissimilar mammalian cells and thus determine the chromosomal location of genes by following the progeny karyotype during subsequent segreation of chromsomes under conditions which were selective for retention of the gene being mapped. The application of fusion methods to the transfer of bacterial plasmid DNA to mammalian cell recipients has previously emphasized the utility of this method for the study of transient expression of genes as compared to the isolation of genetically stable recombinants as a means to permanently alter the genetic characteristics of human recipient cell populations by recombination. This second application of fusion requires the use of mammalian cells that grow well only in media with high levels of serum and limits its applicability to cell types that are much less sensitive both to toxic contaminants commonly found in reagent grade polyethylene glycol (PEG) and to the manipulations of cell membranes required for fusion transfection. In addition, recombinant episomal DNA isolated from vHa-ras transformed NHBE cells contain Alu-positive human sequences that may be required for replication in human cells.

PHS 6040 (Rev. 1/84)

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

George H. Yoakum	Senior Staff Fellow	LHC	NCI
Louise Malan-Shibley	Microbiologist	LHC	NCI
Paul Amstad	Visiting Fellow	LHC	NCI
Curtis C. Harris	Chief	LHC	NCI

Objectives:

The natural occurence of inter and intraspecies transfer of genetic information is well documented among prokaryotes, and there are reports indicating that the natural exchange of genetic information between primates and mice has occured via endogenous type C retroviruses. Therefore, the development and application of genetic methods to transfect human cells grown in tissue culture provides an essential tool for isolation, identification, and study of homologous and heterologous cellular genes. Thus, genetic construction of specialized recombinant human cell lines, developed for the production of cellular, viral, and oncogene products synthesized in vitro by the appropriate human cell type without the use of "helper-viruses", provides for the safe production of biological products for biomedical use and research into the pathologic mechanism of these genes in human cells.

Methods Employed:

High-frequency transfection into human cells required significant modification of the protoplast fusion methods developed formurine, simian, and HeLa cells. Protoplasts were prepared by growth of plasmid-carrying derivatives of HB101 in 250 ml of L-broth to 2 x 10^8 to 5x 10^8 cells per milliliter. Chloramphenicol was added to a final concentration of 200 ug/ml and incubation at 37°C was continued for 18 to 20 hours to amplify the plasmid copy number. After centrifugation, cell pellets were placed on ice and protoplasts were prepared as follows: (i) the pellets were resuspended in 2.5 ml of HBS-20 buffer, (ii) 0.8 ml of freshly mixed lysozyme at 10 ml/ml in HBS-20 was added, and (iii) incubation at room temperature for 15 to 45 minutes was followed by microscopic observation of the conversion of Escherichia coli (E. coli) cells to spheroplasts to determine when reactions were complete. After the lysozyme had converted 85 to 90 precent of the cells to spheroplasts, the mixture was placed on ice, 0.4 ml of 1.25 M $CaCl_2$ was added to stop the lysozyme, and 2.5 ml of 0.25 M EDTA was added to chelate excess Ca^{2+}. This mixture was diluted by slow addition to 12.5 ml of HBS-9 buffer resulting in a preparation containing approximately 2 x 10^9 protoplasts per milliliter.

The fusion procedure was conducted by placing 1.0 ml of 48 percent PEG-1000 preparation in each 60-mm dish containing 5 x 10^4 cells per dish. The culture dishes were centrifuged at 850 g for 3 minutes to approximate protoplasts and human cells. The protoplast supernatant was removed and dishes were flooded with 2.5 ml of 48 percent PEG-1000 fusion reagent prepared as follows: (i) PEG-1000 (polyethylene glycol, Baker grade) was heated to 42°C; (ii) 300 to 500 ml of this melted reagent was poured into a large beaker and; (iii) 10 g of Bio-Rad mixed-bed resing AG501-X8 (D) was added and the mixture incubated for 4 hours at 40° C; (iv) the PEG-1000 was collected by filtering the mixture through Whatman paper No. 1 covered by 20 g of unexposed resing into a vacuum flash; (v) the fusion grade PEG was weighed while still warm and adjusted to a 48 percent solution by weight by the addition of MCDB 151 nutrient medium stock. This PEG-fusion reagent was passed through a 0.22 um filter for sterilization and stored at -20°C for as long as 1 year without notable differences in performance.

Cell and protoplast mixtures were treated with PEG-fusion reagent for 45 to 60 seconds, the mixture was removed, and culture dishes were carefully washed three to five times with MCDB 151 medium to remove most of the residual PEG. Washed cells were covered with LHC-4 growth medium and placed in the incubator; the meidum was changed at 1-hour intervals for the next 3 hours. The LHC-4 culture medium was subsequently changed each morning for the next 3 days. Within 48 to 72 hours after the procedure, transfected cells could be handled normally (for example, trypsinsized for passage). We defined "stable" transfectants for this study as those populations that maintained the selected marker in 80 to 90 percent of the cells after growth for a minimum of ten deivisions without selection.

All other methods employed are standard techniques for recombinant DNA construction, southern hybridization analysis, and gene-product analysis by radioimmunoassay or readioactive labeling and gel electrophoresis after immunoprecipitation with monoclonal antibodies.

Major Findings:

The protoplast fusion transfection method is now optimized for several important human cells: (i) carcinoma (NCI H292/A1146); (ii) human lung fibroblasts; and (iii) normal human bronchial epithelial cells (NHBE). Novel episomal genetic elements have been isolated from vHa ras transformed NHBE cells (TBE-1) with Alusequences vHa-ras sequences; and the ability to "shuttle replicate" in *E. coli*. This provides an opportunity to isolate a segment capable of providing a stable genetic shuttle vector between human and *E. coli* cells.

The frequency of stable transfection in NCI H292 cells with pSV2-derived plasmids was 3 x 10^{-3} after selection for gpt+ or neo+ markers in recipient cells. Cells with the gpt+ marker can be selected on the basis of their ability to convert xanthine to guanine when grown in medium containing aminopterin and mycophenolic acid; the neo+ (neomycin) marker can be selected by growing the cells in medium containing G418-neomycin. The adapted protoplast fusion procedure described

yields substantially higher frequencies of stable transfection into human cells than the CaPO4-DNA method. NCI H292 cells stably carrying the pKYC200 plasmid (GTC2) were isolated by selection for the expression of the gpt+ gene. The transfected genes have remained stably integrated after more than 30 passages of GTC2 cells in RPM1 1640 medium containing 10 percent fetal bovine serum (FBS) (HUT medium).

Protoplast fusion transfection experiments with NHBE cells were performed. Normal human bronchial epithelial cells grown in serum-free medium that can be used as transfection recipients in oncogene studies. Since epithelial cells transfected with oncogenes may have defects in their program for differentiation, it might be possible to select NHBE cells transfected with v-Ha-ras by growing them in the presence of agents that induce differentiation. For example, NHBE cells in LHC-4 medium undergo terminal squamous differentiation when exposed to small amounts of blood-derived serum (BDS) or to 12-O-tetradecanoylphorbol-13-acetate (TPA), whereas v-Ha-ras transfected cells do not. This selection method yielded v-Ha-ras transfected colonies at a frequency of approximately 10^{-3}.

Detection of replicative v-Ha-ras+ episomal DNA from TBE-1 cells. The human DNA preparations isolated from nuclear and cytosol fractions were tested for the presence of episomal DNA by transfection into E. coli (HB101) and selection for Ap^r (ampicillin resistant) colonies. The covalent linkage of pBR322, with a replicative origin recognized by E. coli, and an Ap^r-gene form the H1 plasmid originally transfected by protoplast fusion into NHBE cells, provides a means to select for recombinant episomal DNA. The presence of episomal DNA that contains the functional elements is indicated by the detection of Ap^r HB101 colonies after transfection with TBE-1 DNA preparations.

Recombinant plasmids were isolated for identification and characterization of new sequences not present when plasmid H1 was transfected into NHBE. The recombinant plasmids isolated in this fashion comprise three general size classes with inserts that range from 4Kbp to 12 Kbp. Southern hybridization analysis indicates that approximately 80% of these plasmids isolated from HB101 transfected with TBE-1 DNA contian v-Ha-ras when probed with a ^{32}P-labeled 700 bp Ha-specific fragment probe. In addition, all of the plasmids isolated in this experiment contain Alusequences as indicated by hybridization with a 300 bp Alu specific fragment probe. Thus, TBE-1 cells, after growth in cell culture for more than 200 generations, are stably maintaining episomal forms of the transfected sequences combined with human DNA sequences indicting that (i) genetic recombination between the transfected H1 DNA and naturally occurring human episomal sequences is important to the maintainance of the v-Ha-ras gene; (ii) the covalent linkage of pBR322 replication and Ap^r elements with the human episomal elements is capable of maintaining this "shuttle-vector" property for long periods; and (iii) the segments of human DNA are large enough to contain previously unidentified human gene(s) that may be important to the transformed phenotype of TBE-1 cells.

Significance to Biomedical Research and the Program of the Institute:

The discovery that transfected NHBE cells contain recombinat episomal DNA is of general importance for the following reasons: (i) the mechanism of transformation may be related to maintianing and expressing the v-Ha-ras gene in this fashion; (ii) the human sequences required to replicate the recombinant episomal DNA may provide human cell specific elements needed to construct a stable human cell, *E. coli* shuttle-vector; and (iii) the potential presence of a new human oncogene important to *Ha-ras carcinogenesis* must be considered.

Proposed Course:

The isolation and characterization of recombinant sequences for the following properties: (i) the ability to replicate and maintain plasmid DNA in human cells as an episomal element; and (ii) the presence of secondary oncogenic elements important to the transformation of NHBE cells.

Publications:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05422-01 LHC

PERIOD COVERED
October 1, 1984 to September 30, 1985

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)
Compounds Modifying Growth and Differentiation of Human Esophageal Cells

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	Koji Sasajima	Expert	LHC	NCI
Others:	Susan P. Banks-Schlegel	Senior Staff Fellow	LHC	NCI
	Curtis C. Harris	Chief	LHC	NCI

COOPERATING UNITS (if any)

LAB/BRANCH
Laboratory of Human Carcinogenesis

SECTION
In Vitro Carcinogenesis Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.5	0.5	0

CHECK APPROPRIATE BOX(ES)
☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

By using chemically defined serum-free LHC media, we have investigated the effects of selected compounds on growth and differentiation of human esophageal epithelial cells. The effects were evaluated on clonal growth rate (CGR), i.e., population doublings per day (PD/D), cross-linked envelope (CLE) formation, and the enzymatic activities of ornithine decarboxylase (ODC) and plasminogen activator (PA). In LHC media, the cells did not possess a stratified differentiation, but grew well. CGR was 0.9 ± 0.07 in LHC-8 media and 1.0 ± 0.08 in medium 199 containing 10% fetal calf serum.

We found epidermal growth factor (EGF) was inhibitory to CGR, while it increased ODC activity of cells. Bovine pituitary extract alone increased both CGR and ODC activity. ACTH and beta-endorphin also stimulated CGR with an increased C-AMP level. These compounds did not affect PA activity and CLE formation. The effects of 12-O-tetradecanoyl phorbol-13-acetate (TPA) varied depending on media. TPA was inhibitory to CGR, but induced ODC and PA activities, and terminal squamous differentiation in LHC medium without EGF. Induction of ODC activity and terminal differentiation by TPA were not found in LHC media with EGF.

W-7, an antagonist of calcium binding protein, calmodulin, was inhibitory to CGR (ID50: 8 x 10 to the negative sixth power M) and ODC activity. Fifty percent growth inhibitory dose of TPA was decreased more than 10-fold in the presence of 10 to the negative seventh power M W-7. W-7 blocked induction of ODC activity, and slighly enhanced the induction of terminal differentiation by TPA.

PHS 6040 (Rev. 1/84)

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

Koji Sasajima	Expert	LHC	NCI
Susan P. Banks-Schlegel	Senior Staff Fellow	LHC	NCI
Curtis C. Harris	Chief	LHC	NCI

Objectives:

To clarify the differential effects of tumor promoters on normal human esophageal epithelial cells in culture. To detect the compounds which possibly act as tumor promoters and cocarcinogens in human carcinogenesis.

Methods Employed:

Cell culture: Normal primary human esophageal epithelial cells were cocultivated with irradiated 3T3 fibroblasts and grown in growth media 199 with 10% fetal calf serum and growth supplements (5 x 10^{-7} M hydrocortisone, 10^{-7} M cholera toxin, 5 µg/ml insulin, 5 µg/ml transferrin and 15 ng/ml EGF). In late log phase, cells were subcultured either into media 199 or into serum-free LHC media without feeder cells. LHC-8 medium (MCDB 151 medium supplemented with 10^{-9} M T_3, 5 ng/ml EGF and 35 µg/ml of aqueous extracted bovine pituitary extract [PEX]). LHC-2 was LHC-8 without PEX. In LHC-0, EGF was deleted from LHC-2. For PDBU binding and ODC activity assays, the cells were inoculated onto surface-coated 24 well plates without 3T3 feeder cells.

PDBU binding assay: After cultivation for 24 hour, the cells (10^5) were washed with PBS and then incubated in assay buffer (PBS containing 1 mg/ml BSA) containing 30 nM of [^{3}H]PDBU in the presence or absence of 1 µM unlabeled PDBU at 37°C for 30 minutes. The cells were washed and then solubilized in PBS containing 10% Triton X-100, 1% SDS and 1% NaOH for 30 minutes. The radioactivity of the cell lysate and 2 washings were counted. Specific binding was defined as the difference between total and nonspecific binding. For equilibrium analysis, [^{3}H]PDBU concentration was varied from 5 to 100 nM. Down regulation of [^{3}H]PDBU binding was examined by preincubation with 100 nM unlabeled PDBU for 2 to 6 hours. After removal of bound PDBU by washing with PBS, the specific binding was determined.

Ornithine decarboxylase (ODC) activity assay: The cells (10^5), cocultivated in either medium 199 or LHC media with or without TPA (1 to 100 nM), were incubated for 6 hours at 37°C. ODC activity was measured as the release of $^{14}CO_2$ from labeled ornithine during one hour incubation. Specific activity of ODC was calculated as nmole/hr/mg protein.

Morphological changes: One thousand cells were seeded per 60 mm culture dish in medium 199 containing a 3T3 feeder layer. After incubation for 7 days, the medium was replaced with LHC media or fresh medium 199 and the cells exposed to TPA for 6 hours. Subsequently, the cells were incubated in each 7th respective media without TPA for 24 hours. Then, medium was replaced with medium 199. Morphological changes were judged after incubation for another 2 days by phase contrast microscopy.

Major Findings:

By Scatchard analysis of equilibrium data, esophageal epithelial cells had saturable binding receptors for PDBU. Dissociation constant was calculated to be 24 nM in medium 199. At a saturating concentration of PDBU, around 4.0 p mole/10^6 cells (2.4 x 10^6 binding sites/cell) were bound to cells. These characteristics were almost the same in different media. Specific binding of [^{3}H]PDBU to cells reached a maximum within 60 minutes at 37°C and then decreased significantly at 120 minutes in medium 199 and LHC-0 medium (Student's t-test, $P < 0.005$). In LHC-2 and LHC-8 media, binding radioactivity did not decrease but increased at 120 minutes. Furthermore, preincubation with 100 nM unlabeled PDBU for 2 hours decreased the binding by 30% and 10% in LHC-0 and medium 199, respectively. After preincubation for 6 hours, the binding radioactivity restored in LHC-0, but reduction of binding was increased to 30% in medium 199. The cells did not show down regulation of receptors in LHC-2 and LHC-8.

Effects of TPA and ODC activity varied depending on the media in which the cells were grown. TPA (10 and 100 nM) induced ODC activity of cells to 190% and 240% in medium 199 and LHC-0 medium, respectively ($P < 0.005$). In LHC-2 and LHC-8 media, TPA was inhibitory to ODC activity.

A stratified differentiation of esophageal cells was only found when they were cultured in medium 199. After replacement of medium from serum-free LHC media to medium 199, cells showed stratified differentiation and grew well. When cells were exposed to TPA (1 to 100 nM) for 6 hours in medium 199 and LHC-0 medium, TPA (100 nM) induced morphological terminal squamous differentiation.

Significance to Biomedical Research and the Program of the Instute:

We have investigated the characteristics of [^{3}H]PDBU binding to normal human esophageal epithelial cells and found the down regulation of receptors and EGF may play an important role in tumor promotion. This system is useful for the detection of compounds which may act as tumor promoter on esophageal carcinogenesis. These investigations may help to identify the biochemical mechanism of tumor promotion.

Proposed Course:

The characterization of [^{3}H]PDBU has been completed in cancer cell lines. Selected compounds will be examined on [^{3}H]PDBU binding to normal esophageal cells.

Publications

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05423-01 LHC

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Specific Binding of [3H] Phorbol 12,13-Dibutyrate to Human Esophageal Cells

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Koji Sasajima	Expert	LHC	NCI
Others:	Susan P. Banks-Schlegel	Senior Staff Fellow	LHC	NCI
	Curtis C. Harris	Chief	LHC	NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Human Carcinogenesis

SECTION

In Vitro Carcinogenesis Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.5	0.5	0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Tumor promotion has been shown to play an important role in the etiology of human cancer. However, there are interspecies and intertissue variations in the response to tumor promoters. The effects of a potent tumor promoter, 12-0-tetradecanoyl phorbol-13-acetate (TPA) on human epithelial cells vary depending on culture conditions. TPA induced terminal squamous differentiation and ornithine decarboxylase (ODC) activity on human esophageal epithelial cells cultured in medium 199 containing 10% fetal calf serum and in serum-free LHC media without epidermal growth factor (EGF). These effects were not found in LHC media containing EGF. Therefore, we characterized the binding of [3H]phorbol 12,13-dibutyrate (PDBU) to esophageal cells. Specific binding of [3H]PDBU to cells reached a maximum within 60 minutes and decreased at 120 minutes in medium 199 and LHC-0 at 37°C. Furthermore, preincubation of cells in media containing unlabeled PDBU caused reduction in binding (down regulation). By equilibrium analysis, dissociation constant was 24 nM and there were around 2.4 x 10 to the sixth power receptors per cell in medium 199. These characteristics were almost the same in different media. However, binding radioactivity did not decrease after preincubation with unlabeled PDBU in LHC media containing EGF. Results suggest that down regulation may be important for the development of TPA effects on esophageal cells. EGF inhibited down regulation of receptors in serum-free LHC media.

PHS 6040 (Rev. 1/84)

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

Koji Sasajima	Expert	LHC	NCI
Susan P. Banks-Schlegel	Senior Staff Fellow	LHC	NCI
Curtis C. Harris	Chief	LHC	NCI

Objectives:

Using improved culture media and technique, the growth and differentiation of normal human esophageal epithelial cells will be analyzed. These studies are designed to evaluate the essential changes that occur during mitogenesis and malignant transformation.

Methods Employed:

Normal primary human esophageal cells were cocultivated with irradiated 3T3 fibroblasts and grown in medium 199 with 10% fetal calf serum and growth supplements. In late log phase, the cells were subcultured either into medium 199 or into serum-free LHC media without feeder cells. LHC-8 medium (MCDB 151 medium supplemented with 10^{-9} M T_3, 5 ng/ml EGF and 35 g/ml of bovine pituitary extract [PEX]). LHC-2 was LHC-8 without PEX. In LHC-0, EGF was deleted from LHC-2. The growth and differentiation were assessed by morphological changes, and measurement of clonal growth rate (CGR), cross-linked envelope (CLE) formation and the enzymatic activities of ornithine decarboxylase (ODC) and plasminogen activator (PA).

Major Findings:

Effects of media in normal human esophageal epithelial cells in culture were studied. Morphologically, a stratified differentiation of cells was only found in medium 199 containing 10% fetal calf serum. However, the cells grew well in serum-free LHC media. CGR was 0.9 ± 0.07 in LHC-8, 0.5 ± 0.04 in LHC-0 and 1.0 ± 0.08 in medium 199. EGF was inhibitory to CGR of esophageal cells (ID_{50}: 15 ng/ml in LHC-0), while it increased ODC activity. PEX alone increased both CGR and ODC activity. ACTH and β-endorphin also stimulated CGR with an increased C-AMP level. However, these compounds did not affect PA activity and CLE formation.

Differential effects of 12-O-tetradecanoyl phorbol-13-acetate (TPA) in esophageal cells were found. The effects of TPA (1 to 100 nM) varied depending on the culture conditions. TPA was inhibitory to CGR (ID_{50}: 10 nM in LHC-8 medium), but induced ODC (240% at 100 nM) and PA activity, and terminal squamous differentiation in LHC-0 medium. Induction of ODC activity and terminal differentiation by TPA were not found in media LHC-2 and LHC-8.

W-7, an antagonist of calcium binding protein, calmodulin, was inhibitory to CGR (ID_{50}: 8×10^{-6} M in LHC-8 medium). On ODC and PA activity, W-7 did not affect at relative low concentration (10^{-7} M) and decreased at 10^{-5} M in both medium 199 and LHC-8 medium. When the cells were incubated with W-7 (10^{-7} M), 50% growth inhibitory dose of TPA was decreased more than 10-fold. W-7 (10^{-7} M) blocked the induction of ODC activity and slightly enhanced terminal differentiation by TPA in medium 199.

Significance to Biomedical Research and the Program of the Institute:

This system will provide information regarding the growth and differentiation of normal human epithelial cells.

Proposed Course:

A role of EGF on growth and differentiation of esophageal cells will be completed. Further studies include the following: (1) understand the regulation of normal differentiation, (2) evaluate the role of calcium ion; and (3) identify unknown growth stimulating factor in PEX.

Publications

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05424-01 LHC

PERIOD COVERED
October 1, 1984 to September 30, 1985

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)
The Role of Respiratory Viruses in Lung Carcinogenesis

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	Roger R. Reddel	Guest Researcher	LHC	NIH
Others:	Brenda I. Gerwin	Research Chemist	LHC	NIH
	Curtis C. Harris	Chief	LHC	NIH

COOPERATING UNITS (if any)
Dept. of Immunology and Infectious Disease, Johns Hopkins Unviersity, School of Hygiene and Public Health (K. Shah and C. Christian); Endwood Division of Infectious Diseases Dept. of Pediatrics, The Johns Hopkins Hospital, Baltimore, MD (B. Maldonads and R. Yolken).

LAB/BRANCH
Laboratory of Human Carcinogenesis

SECTION
Carcinogen Macromolecular Interaction Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.0	1.0	0.0

CHECK APPROPRIATE BOX(ES)
☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

This is a new project to assess the role of respiratory viruses in the multistage process of lung carcinogenesis. Preliminary results indicate that adenoviruses and papilloma viruses infect normal human bronchial epithelial cells in vitro. The long-tem consequences of these infections on the neoplastic potential of the epithelial cells and the possible differential cytotoxic response between normal and neoplastic lung cells is being studied.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

Roger R. Reddel	Guest Researcher	LHC	NCI
Brenda I. Gerwin	Research Chemist	LHC	NCI
Curtis C. Harris	Chief	LHC	NCI

Objectives:

To describe the effects of infection of human bronchial epithelial cells with selected human respiratory viruses; to investigate whether viral infection or transfection of viral DNA may cause increased life span or full transformation of normal human bronchial epithelial cells or full transformation of HBE which have been immortalized by other agents but which do not exhibit the full spectrum of behavior of malignant cells; to develop an in vitro culture system for human papillomavirus (HPV).

Methods Employed:

This study employs a number of techniques developed in this laboratory including culture of normal human bronchial epithelial cells from explanted autopsy tissue, treatment of these cells with inducers of differentiation and transfection of plasmid DNA via protoplast fusion. In addition, nontumorigenic transformed bronchial epithelial (TBE) cells have been utilized, as well as several readily available human lung carcinoma cell lines. Cell morphology following viral infection has been followed with light and electron microscopy and immunofluorescence techniques. Release of viral antigens into the culture medium has been monitored using ELISA techniques. Progression of cells to the malignant phenotype is monitored by survival past senescence of control cultures, soft agar cloning, karyology and injection of cells into nude mice. Viral genes reported to cause cellular transformation in other experimental systems have been subcloned into the pSV2neo vector. Viral DNA in extracts of infected cells has been detected by hybridization to nick-translated cloned viral DNA.

Major Findings:

Preliminary results include: (1) adenoviruses 5 and 12 cause persistence of infected HBE cells for several months beyond the time of senescence of control cultures; the infected cells, however, do not appear to be proliferating; (2) replication of HPV DNA appears to occur in HBE cells and, rarely, viral capsid proteins are expressed.

Significance to Biomedical Research and the Program of the Institute:

Further work is required to assess the significance of the extended life span observed in adenovirus-infected cells. In a number of experimental systems it has previously been observed that transformation does not occur in productively-infected cells. The delayed senescence may simply represent virally induced resistance to terminal differentiation in cells which are unable to proliferate.

It remains to be determined whether a full HPV infection cycle can be supported by NHBE cells in culture. HPV DNA replication has been observed previously in a number of other cell culture systems, but no in vitro model of the full infection cycle exists. Preliminary experiments using an avidin-biotin stain for HPV capsid antigens indicate the possibility that HPV virione may be assembled in NHBE cells.

Proposed Course:

Representative respiratory viruses are being used to infect NHBE cells, nontumorigenic TBE lines and lung carcinoma cell lines to see whether there are differences in susceptibility of these different cell types to viral infection. In addition, any of the NHBE or TBE cells which continue to replicate after viral infection will be screened for their ability to grow in soft agar and in nude mice.

The presence of HPV capsid proteins in infected NHBE cells will be further investigated by Western blotting of cellular protein extracts. Inducers of HBE cell differentiation, e.g., TGF-β or TPA, are being used to attempt to maximize vegetative virus production.

The pSV2-neo constructs containing adenovirus and CMV genes are being used to transfect HBE cells to determine whether immortalization or the fully transformed phenotype can be induced.

Publications:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05425-01 LHC

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Asbestos Carcinogenesis/Chromosomal Changes in Human Cells

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	Kaija Linnainmaa	Visiting Fellow	LHC	NCI
Others:	Brenda Gerwin	Research Chemist	LHC	NCI
	Edward Gabrielson	Medical Staff Fellow	LHC	NCI
	Curtis C. Harris	Chief	LHC	NCI

COOPERATING UNITS (if any)

Centers for Disease Control, Atlanta, GA (J. Lechner)

LAB/BRANCH

Laboratory of Human Carcinogenesis

SECTION

In Vitro Carcinogenesis Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.5	1.0	0.5

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Asbestos-induced chromosomal abnormalities have been studied in human pleural mesothelial cells and in bronchial fibroblast cultures in vitro. After one exposure to amosite, a significant increase in chromosomal aberrations was found in mesothelial cells, as compared to unexposed controls. The number of aberrant cells was further increased in cultures treated twice with asbestos, the major aberration type being dicentric chromosomes and acentric fragments (minute chromosomes). The number of aneuploid cells was observed to increase in all the mesothelial cell cultures with the passage number of the culture. However, no significant differences were found between the asbestos-exposed and control mesothelial cells. In asbestos-exposed fibroblasts, on the other hand, the number of aneuploid cells was significantly elevated, as compared to unexposed controls.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

Kaija Linnainmaa	Visiting Fellow	LHC	NCI
Brenda Gerwin	Research Chemist	LHC	NCI
Edward Gabrielson	Medical Staff Fellow	LHC	NCI
Curtis C. Harris	Chief	LHC	NCI

Objectives:

The aim of this project is to study the induction of chromosomal abnormalities and their relationship to carcinogenic processes in asbestos-exposed human pleural mesothelial cells and in bronchial fibroblast cultures in vitro. Studies are directed to follow the occurrence of chromosomal abnormalities in several subsequent passages of the asbestos-exposed cell cultures as well as their nonexposed control cultures. Both chromosomal aberrations and numerical chromosomal changes will be analyzed. Karyotypic analyses will be carried out in order to examine the development of possible consistent chromosomal changes in phenotypically altered colonies of the asbestos-exposed cell cultures.

Methods Employed:

The methods for culturing human pleural mesothelial cells and bronchial fibroblasts, as well as a protocol for an in vitro asbestos-carcinogenesis experiment have been developed in this laboratory previously. The cells are exposed in two subsequent doses of amosite asbestos (2 μg/ml), and the cultures are subcultured and carried on as long as possible looking for evidence of phenotypically altered cells. Concurrent control cultures are carried out in parallel. For chromosomal preparations the mesothelial cell cultures are first partly synchronized in G1 stage. Exponentially growing cells are treated with colcemid to accumulate the mitotic cells in metaphase and the harvested cells are treated with hypotonic solution, fixed in acetic acid-methanol mixture and dropped on slides. Slides are stained with Giemsa for aberration analyses and trypsin-Giemsa method is applied for the karyotypic analyses.

Major Findings:

After one exposure to amosite, a significant increase in chromosomal aberrations including chromosome and chromatid breaks was observed in mesothelial cell cultures, compared to untreated control cells. The number of aberrant cells was further increased in cultures treated twice with asbestos, the major aberration types being dicentric chromosomes and acentric chromosome fragments (minute chromosomes). The number of hypodiploid cells was observed to increase with the passage number of the culture. No significant diffferences were, found, however, between the asbestos exposed cultures and the corresponding control cultures. Similar results have been obtained from two repeated experiments. The karyotypic analyses of the cells from the latest passages of the two experiments (passages 3 and 5) are underway. The phenotype of the asbestos-exposed mesothelial in their fourth subculture was observed to differ significantly from the unexposed control cultures. The control cultures of

the first experiment reached senescence growth during the fourth subculture. One phenotypically altered colony has been found so far in the asbestos-exposed cultures. The generation time of these cells appears to be significantly greater than that of early passage cells.

Unlike the mesothelial cell cultures, the number of hypodiploid cells was significantly elevated in the asbestos-treated fibroblast cultures as compared to untreated controls.

Significance to Biomedical Research and the Program of the Institute:

Asbestos is considered to be the major cause of mesothelioma and a cocarcinogenic factor in the etiology of bronchogenic carcinoma. These studies will provide information of the mode of initiation of carcinogenesis by asbestos in human target cells.

Proposed Course:

The karyology of the cells from phenotypically altered colonies will be followed as long as the cells continue to multiply. If consistent abnormalities (e.g., marker chromosomes or minute chromosomes) are found, a possible role of oncogene activation will be studied by in situ hybridization technique.

Publications

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01CP05426-01 LHC

PERIOD COVERED
October 1, 1984 to September 30, 1985

TITLE OF PROJECT (*80 characters or less. Title must fit on one line between the borders.*)
Characterization, Mode of Action, and Evolution of the Oncogene Raf

PRINCIPAL INVESTIGATOR (*List other professional personnel below the Principal Investigator.*) (*Name, title, laboratory, and institute affiliation*)

PI:	George E. Mark, III	Expert	LHC	NCI
Others:	Dimitrios Boumpas	Visiting Fellow	LHC	NCI
	Paul Amstad	Visiting Fellow	LHC	NCI
	Dean L. Mann	Mecial Officer	LHC	NCI
	Curtis C. Harris	Chief	LHC	NCI
	Mike R. Blaese	Medical Officer	MET	NCI

COOPERATING UNITS (*if any*)
Arthritis and Rheumatism Branch, NIADDKD, NIH (J.D. Mountz and M.F. Seldin); Dept. of Human Genetics, Roswell Park Memorial Institute (T.B. Shows); V.A. Medical Center, Syracuse, NY (B.J. Poiesz).

LAB/BRANCH
Laboratory of Human Carcinogenesis

SECTION
Carcinogen Macromolecular Interaction Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.7	0.5	0.2

CHECK APPROPRIATE BOX(ES)
☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (*Use standard unreduced type. Do not exceed the space provided.*)

We have investigated the expression of the c-raf-1 proto-oncogene in neoplastic as well as hyperplastic pathologies. All small cell lung carcinomas examined express elevated levels of raf transcripts, this was particularly true of non-cultured metastases. Characterization of other surface markers revealed the presence of early monomyelocyte antigens, suggesting that these cells may have evolved from hemopoietic precursors. Activated T and B cells obtained from the peripheral blood of human autoimmune dieased individuals or from lymph nodes and spleens isolated from autoimmune mice, also expressed elevated levels of the raf proto-oncogene transcript. A human fetal liver cDNA library was screened at reduced stringency for v-raf related sequences. In addition to the expected c-raf-1 cDNA a novel sequence was isolated. Comparison of the new gene (c-pks-1) to the other raf homologs revealed nucleotide homolgies of 71%. The expression of c-pks-1 mRNA (2.8-Kb) is elevated in peripheral blood mononuclear cells isolated from patients with systemic lupus erythematosus and angioimmunoblastic lymphadenopathy with dysproteinema (AILD). In the course of localizing the c-pks-1 gene to the short arm of the X chromosome (Xpter-Xp11), another related gene (c-pks-2) was recognized and localized to chromosome 7 (7pter-7q22). The yeast homolog of raf has been cloned into λgt 10 and subcloned into pBR322. Sequencing and expression studies are planned.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on This Project:

George E. Mark III	Expert	LHC	NCI
Dimitrios Boumpas	Visiting Fellow	LHC	NCI
Paul Amstad	Visiting Fellow	LHC	NCI
Dean L. Mann	Medical Officer	LHC	NCI
Curtis C. Harris	Chief	LHC	NCI
R. Michael Blaese	Chief, Cellular Immunol. Sect.	MET	NCI

Objectives:

Of the four major classes of oncogenes the largest class contains the members of the src family. These genes, many of which have been shown to possess either a tyrosine of serine/threonine kinase activity, are found associated with the cell membrane and are believed to be components of the cell's receptor system. It is the stimulation of one or more of these receptors which initiates a cascade of events leading to cellular proliferation. The objectives of this project are (1) to identify and characterize new proto-oncogenes so that additional potential targets of transforming lesions might be enumerated along with loci involved in normal cellular growth and differentiation, and (2) to determine what human disorders may involve the inappropriate expression of the proto-oncogenes related to raf in the hope that the pathology may illuminate the physiologic role of these genes.

Methods Employed:

RNAs were isolated from tissue culture cells, mouse organs or human peripheral blood mononuclear cells by the guanidinium isothiocyanate-CsCl method of Chargwin and poly (rA) containing transcripts were selected by oligo dt-cellulose chromatography. Analysis of RNAs was either quantitative dot blotting or qualitative Northern analsysis followed by hybridization with nick-translated 32-P labelled probes. A human fetal liver cDNA library, made in λgt 10, was obtained from Dr. Ed Fritch (Genetics Institute, Boston, MA) and screened for v-raf related phage by conventional methods after reducing the stringency of hybrid selection. Eco RI digested yeast DNA of approximately 4.5-Kb was extracted from Seaplaque agarose, ligated into λgt 10, and screened as described above. Nucleic acid sequencing protocols, described by Maxam and Gilbert, for the chemical cleavage of terminally radiolabelled oligonucleotides were employed. Surface marker studies were performed on the FACS using established fluorescent labelled monoclonal antibody procedures.

Major Findings:

The relationship between oncogenes and small cell lung carcinomas, pancreatic carcinomas and lymphoproliferative disease (systemic lupus erythematosus) is under examination. The c-raf-1 proto-oncogene, a unique member of the src family as a consequence of its intrinsic serine (not tyrosine) kinase activity, has been localized to the short arm of human chromosome 3, proximal to deletions observed in most small cell lung carcinomas. Cells derived from this particularly agressive type of lung cancer have been shown to contain amplified

expression of one of the myc proto-oncogenes (either c-myc, N-myc, or L-myc). This may explain the state of differentiation of these cells but not their proliferative capacity.

To determine the presence and relative amounts of c-raf-1 (subseqeuntly referred to as raf), specific transcripts in small cell lung carcinomas (SCLC) total cellular RNA was isolated from numerous established cell line. Diluted RNAs were dot blotted onto nitrocellulose filters and hybridized with a human raf probe. All of the SCLC cell lines were positive for raf expression, most showing levels of expression $\geq$ 20-fold greater than that found in normal bronchial epithelial cells. This amplified state of transcription was not dependent upon the existence of an amplified myc proto-oncogene, since no significnt difference was found in raf expression in these cells relative to cells in which myc was not amplified. RNAs isolated from fresh lung paranchyma or normal liver obtained by autopsy, or non small cell lung carcinomas obtained by biopsy, revealed little, if any, raf expression. The only normal tissue to consistantly show raf transcripts was of lymphoid origin.

Analysis of SCLC liver metastases obtained from two patients at autopsy revealed high levels of raf expression, 10- to 25-fold more than was seen in the cell lines. Thus, we believe that amplified levels of raf expression may lead to uncontrolled proliferation in SCLC. The observation of significnt raf expression in lymphoid cells led us to investigate the surface markers on the established SCLC cell lines. Employing fluorescence conujuated monoclonal antibodies and the FACS, the surface characteristics of various cell lines were determined.

The macrophage/monocyte antigen detected by OKM1 has been previously shown to be on the surface of SCLC cells (Ruff and Pert, Science 225: 1034-1036, 1985) and this observation was interpreted to point toward a myeloid or hemopoietic stem cell as the target of transformation. Although most of the SCLSs examined express more of this antigen (the C3bi complement receptor) than the other lung carcinomas (with the exception of the H292 cell line), only 5 of the 8 are higher than the normal bronchial epithelial cells. The striking finding of our study is the unique presence of two early monomyelocytic markers, MY4 and MY9, on the SCLC cells. It is tempting to conclude that small cell lung carcinomas are in actuality lymphomas whose stem cell was an immature monomyelocyte.

Systemic autoimmune disease states are known to be associated with abnormal cell growth and/or differentiation. Systemic lupus erythematosus (SLE) is the prototype human autoimmune disease characterized by T and B cell dysregulation. Models for this syndrome are available in the mouse, where classical genetics have identified several autoimmune genes (lpr, gld, and xid); as yet the molecular nature of these genes is not known. High expression of myb and raf are found in the lymph nodes of lpr/lpr mice, while subnormal amounts of myb are evident in the thymus of these animals. These and other studies suggest that associated with the lpr impairment is an inability of T cells to differentiate, hence they are believed to be frozen in a stage of development characterized by myb expression and concurrent accumulation in lymph nodes leads to the ensuing lymphadenopathy. In contrast to myb, raf RNA was decreased in the spleens of xid:lpr/lpr mice but not in their lymph nodes. It is concluded that raf expression is primarily associated with lpr/lpr B cells in spleens and T cells in lymph nodes. These studies were extended by examining the

peripheral blood mononuclear cells from patients with systemic lupus erythematosus (SLE) and other autoimmune diseases, these patients were found to have a signifcantly increased level of the c-myc, c-myb and c-raf proto-oncogene RNAs (10- to 50-fold) compared with normal individuals. Where paired samples were available, proto-oncogene expression correlated directly with disease activity in that it was higher during the exacerbation of the disease. These findings, we believe, reflect the pathologically activated state of lymphocytes observed in SLE and other autoimmune diseases and suggest a role for the activation of these proto-oncogenes in the increased incidence of lymphoproliferative disorders observed in patients with autoimmune diseases. We also suggest that this implicates the raf proto-oncogene in the normal proliferative mechansism of hemopoietic cells.

The DNA and protein sequences of c-pks-1 indicated that this gene was closely related to the c-raf-1 gene. Normal peripheral blood mononuclear cells express very little of either sequence. Extremely high expression of only the c-pks-1 gene was found in a patient with angioimmunoblastic lymphadenopathy with dysproteinema (AILD). This disease is characterized by the lymphoproliferative activation of B cells as a consequence of dysfunctional T cells producing lymphokines, leading to the production of polyclonally-derived auto-antibodies. The lack of c-raf-1 mRNA suggests that the proliferative inbalance associated with AILD is different than that exhibited by another autoimmune disease (SLE).

Most proto-oncogenes have been highly conserved through evolution since the appearance of metazoan organisms; hence, the belief that their role in normal cellular development and differentiation has transgressed time unaltered. A Drosophila melanogaster genomic library, made from size selected partial Eco RI digested DNA ligated into the λ phage Charon 4A, was screened with a 32-P labelled v-raf probe under relaxed stringency conditions. In order to establish, unambiguously, the relationship of the D-raf-1 locus to the raf oncogene, the DNA sequence of 0.95 Kb pst 1 fragment was determined. Alignment of the D-raf-1 sequence with that of v-raf revealed the presence of a 65 nucleotide insertion in the Drosophila sequence. This inserted sequence is flanked by cononical splice donor and acceptor sites and must be removed post-transcriptionally; otherwise, translation would be terminated. The overall homology of D-raf-1 to v-raf is 61%. The predicted amino acid sequence of D-raf-1 indicates it is a raf homolog and as such probably provides identical functions in fruit flies as the related genes do in man and mouse. Various domains, invisible in the mouse-human comparison, are distinguishable within the polypeptide. Those sequences preceeding the nucleotide binding region (amino acid positions 14 to 35) and following amino acid position 224 are poorly conserved relative to those sequences which lie between these two domains. Interestingly, the region of highest homology ends at the sequence M-V-G-R-G which has been found in the ras gene family, G protein family (including the transducing subunit), and as an integral part of the nucleotide binding site in the elongation factor Tu. This may represent the end of those sequences which comprise the nucleotide binding domain, essential for the kinase activity of these proteins.

Using the Drosophila gene (D-raf-1 0.95% Kb Pst I fragment) as a probe and lowering the stringency of hybridization as a unique Eco RI fragment (4.5 Kb), Hind III fragments (6.0 and 3.0 Kb) were detected in the yeast genome. This DNA was employed to construct a phage library in λgt 10. Sixteen positive clones have been picked and are being analyzed for hybridization to the D-raf-1 probe.

The initial screening of a human fetal liver cDNA library for raf homologs was performed under conditions of low hybridization stringency. Two non-identical clones were isolated from this λgt 10 library. Restriction enzyme mapping showed one of these clones represented a cDNA copy of a portion of the c-raf-1 gene, while the other was unique. DNA sequencing of this unique gene revealed it was a new raf-related human locus (to be called c-pks) The first 890 nucleotides of c-pks-1 exhibit a 70.5% and 72.0% homology with the DNA sequences of c-raf-1 and v-raf, respectively. No insertions or deletions were seen, suggesting that c-pks-1 was not a pseudogene. The DNA homology to the known raf related genes ends with the termination codon of c-pks-1, approximately 250 nucleotides before the termination of DNA homology between v-raf and c-raf-1. Comparison of the protein sequence of c-pks-1 to that of the raf homologs reveals a homology of 75%. The similarity is even more striking when one considers that at least 34 of 71 amino acid differences between these two sequences are conservative changes. Taking this into account this region of these proteins are 88% homologous. The carboxy end of c-pks-1 appears truncated relative to the other members of this subfamily. This removes the C-T-L-sequence common to the others, a sequence believed to be acceptor for fatty acid esterification.

To determine the number of c-pks genes in the human genome, Southern blot analyses were performed on DNAs from mouse x human somatic cell hybrids. Hybridization to restricted human DNA under conditions of high stringency revealed strong hybridization to the three Bgl II fragments which co-segregated indicating that they represented the cloned c-pks-1 gene. The largest Bgl II fragment hybridized poorly under high stringency to the middle and 3' untranslated region probes. We conclude that there are two closely related c-pks genes in man. Employing extensive analysis of many mouse x human somatic cell hybrids we have localized the c-pks-1 gene to the short arm of the X chromosome (Xpter-Xp11) and the other gene (c-pks-2) to chromosome 7 (7pter-7q22). Thus, the c-pks-1 gene represents the only known active proto-oncogene sequence on a sex chromosome.

Significance to the Biomedical Research and the Program of the Institute:

The isolation and characterization of the oncogene v-raf, its homologs in primate, avian, Drosophila, and Saccharomyces, as well as related gene in man, has resulted in the capability of studying the function and evolution of essential genes controllng cellular proliferation. The demonstration of an association between increased proto-oncogene expression and murine and human autoimmune disease may help identify cells that act in this complex disease process. Observing the c-raf-1 proto-oncogene in SCLC has led to subsequent studies which suggest the malignant cell has numerous characteristics in common with hemopoietic tissue.

Two new human proto-oncogenes has been identified which is closely related to the oncogene raf. Several X-linked lymphoproliferative and immunodeficiency diseases have been sublocalized to presumed defects on the short arm (the p arm) of this chromosome. Hypothetically, c-pks-1 may be altered in such a manner so that this proliferative signal is continual, leading to a lymphoproliferative syndrome. On the other hand, this gene might be made inoperative resulting in the inability of an immature hemopoietic cell to differentiate and expand, leading to an immunodeficiency syndrome.

Proposed Course:

Future work with the c-pks-1 gene will involve the isolation or generation of additional molecular biological tools with which the function of this gene can be studied. Libraries are being screened for complete cDNA representations of the transcript so that the promoter region of the cistron can be examined. Existing cDNA fragments are being introduced into prokaryotic expression vectors in order to generate specific polypeptides which will be used for anitbody production. Both of these approaches will lead to a better understanding of the c-pks-1 protein and its function. Concurrently, tissues from patients with both X-linked lymphoproliferative and immunodeficiency disorders are being analyzed for DNA alterations (gross deletions, amplifications, or insertions) and, where possible, RNA expression. We hope to determine whether c-pks-1 is the cause of any of these diseases.

Investigations of the role of raf in SCLC and autoimmune diseases are being continued from two levels. First, various domains of the proto-oncogene are being cloned into a prokaryotic expression vector. The expressed proteins will be used to develop specific antibodies so that gene products may be identified, on the one hand, or their activities interferred with by antibody microinjection. Second, several oncogenes have been introduced into a Zip vector so that in one orientation the resulting retrovirus will be capable of gene transformation (myb, raf, myc, and ras), while in the opposite orientation (bym, far, cym, and sar) transcription may result in the hybrid arrest of the existing endogenous complementary sequence. We are interested in determining whether the expression of one oncogene will lead to the expression of other proto-oncogenes. Use of Zip vectors allows for the introduction of a specific gene into all of the cells under study. They also facilitate the infection of animals in the absence of a helper virus whose presence may lead to unwanted spread or unnecessary recombinational events. These constructs, when in the opposite orientation, may be able to specifically deminish, or eliminate, the expression of one particular gene so that the role of that sequence in the existing pathology of a cell may be determined. Future work with the raf homologs will focus on exploiting the genetic tools available in Drosophila and Saccharomyces biology.

Publications:

Bonner, T. I., Kerby, S. B., Sutrave, P., Gunnell, M. A., Mark, G., and Rapp, U. R.: The structure and biological activity of the human homologues of the raf/mil oncogene. Mol. Cell. Biol. (In press)

Boumpas, D. T., Tsokos, G. C., Mann, D. L., Eleftheriades, E. G., Harris, C. C., and Mark, G. E.: Increased proto-oncogene expression in peripheral blood lymphocytes from patients with Systemic Lupus Erythematosus and other autoimmune diseases. Lancet (In Press)

Mark, G. E., Seeley, T. W., Shows, T. S. and Mountz, J. D.: C-pks, a new raf related sequence in man. Science (In Press)

Mountz, J. D., Mushinski, J. F., Mark, G. E., and Steinberg, A. D.: Oncogene expession in autoimmune mice. J. Mol. Cell. Immunol. (In Press)

Rosenberg, Y. J., Malek, T. R., Schaeffer, D. E., Santoro, T. J., Mark, G. E., Steinberg, A. D., and Mountz, J. D.: Unusual expression of IL2 receptors and both the c-myb and c-raf oncogenes in T cell lines and clones derived from autoimmune MRL-lpr/lpr mice. J. Immunol. 134: 3120-3123, 1985.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05427-01 LHC

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Human Chorionic Gonadotropin as a Marker and Growth Factor in Human Lung Tumors

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	Doug Brash	Senior Staff Fellow	LHC	NCI
Others:	George Yoakum	Senior Staff Fellow	LHC	NCI
	George Mark	Expert	LHC	NCI
	Curtis C. Harris	Chief	LHC	NCI

COOPERATING UNITS (if any)

Dept. Path., Univ. MD School of Medicine, Baltimore, MD (T.S. Wilson, E.M. McDowell, B.F. Trump); Dept. Anatomy, Boston Univ. Sch. Med., Boston, MA S.P. Sorokin; Litton Bionetics, Inc., Rockville, MD (M.G. Valerio)

LAB/BRANCH

Laboratory of Human Carcinogenesis

SECTION

Carcinogen Macromolecular Interaction Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

We have immunohistochemically examined a large number of normal, hyperplastic, metaplastic and neoplastic tissues for presence of human chorionic gonadotropin (α and β HCG). The hormone was present in normal fetus and placenta and in more than 75% of adult lung neoplasms (except SCLC) but not in normal adult tissues. It was also synthesized in tumors generated in athymic nude mice by v-Harvey ras transformed bronchial epithelial cells. Addition of purified α and β HCG subunits to normal human bronchial epithelial cells revealed that α plus β, but neither, alone, is a growth factor for these bronchial cells. These results indicate that HCG is a consistent marker for human lung tumors and that it may play a causal role in lung tumorigenesis as a growth factor for human bronchial epithelial cells. To examine these findings at the level of gene expression, we constructed probes for α- and β-HCG mRNA expression and have identified human lung tumor cell lines which synthesize only one or the other HCG subunit. Human lung tumor cell lines do not detectably differ from normal bronchial epithelial cells in the state of methylation of the HCG genes.

PHS 6040 (Rev. 1/84)

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

C. C. Harris	Chief	LHC	NCI
G. Yoakum	Senior Staff Fellow	LHC	NCI
G. Mark	Senior Staff Fellow	LHC	NCI
D. Brash	Senior Staff Fellow	LHC	NCI

Objectives:

To determine whether ectopic HCG production is a consistent marker in human lung tumors. To determine whether α and β subunits of HCG are growth factors for bronchial epithelial cells. To determine whether the regulation of HCG genes expressed in lung tumors differs from that of genes expressed in the normal placenta.

Methods Employed:

We have developed a rabbit anti-β-HCG/peroxidase antiperoxidase assay for visualizing HCG synthesis in fixed slides of tissue specimens. We have also adapted a published HCG extraction procedure for assaying intracellular α- and β-HCG using an RIA assay. The latter is also used with cell culture supernatants to detect α- and β-HCG secreted by cells in culture. Cultured cells are grown in various media, but normal human bronchial epithelial cells are grown in serum-free, defined media after outgrowth from autopsy explants by methods developed in our laboratory over the last several years. Growth rate of normal bronchial epithelial cells in response to putative growth factors is measured in this culture system. HCG mRNA is detected by using α- and β-HCG genomic and cDNA clones, respectively, as probes. DNA methylation is measured by comparing restriction digestion of HCG genes by restriction enzymes sensitive or insensitive to 5-methylation of cytosine.

Major Findings:

This project has led to new findings in the areas of (1) HCG synthesis by human lung tumors, metaplastic and hyperplastic tissues and normal tissue and (2) effect of HCG as a growth factor for human bronchial epithelial cells, and has established a cellular and molecular system for investigating, in the future, the possible contributing role of HCG in lung tumorigenesis.

HCG was present in fetal human, hamster, rat and guinea pig tracheobronchial epithelia but was not detectable from normal adult tissues. HCG was not present in mucous cell hyperplasia, stratification or epidermoid metaplasia. In contrast, HCG was present in more than 75% of non-SCLC lung tumors and in some intra-epithelial neoplasms. Transfection of normal human bronchial epithelial cells with the v-Harvey-ras oncogene results in transformation. Injection of these transformed bronchial epithelial cells into nude mice results in tumors. Tumors derived from transformed cell line TBE-1SA were stained for β-HCG and found to be positive. To establish an in vitro system for determining the role of HCG in transformation, we assayed a number of lung tumor cell lines for α- and β-HCG

secreted into the medium. Line A1146 produces and secretes α- but not β-HCG, whereas SW900 and CaLu-1 produce β but not α. Secretion can depend heavily on the culture medium used.

Several cell lines derived from v-Ha-ras transfected bronchial epithelial cell lines were also examined for HCG expression. These cells do not make or secrete α-HCG. Activation, therefore, apparently occurs during development of the tumor. To determine whether HCG, α-HCG or β-HCG are growth factors for normal human bronchial epithelial cells, purified hormone or individual subunits were added to NHBE cultures and growth rate determined. Neither subunit alone stimulated growth of NHBE cells, but when both subunits were added together to form HCG, human chorionic gonadotropin was a growth factor for normal human bronchial epithelial cells.

Significance to Biomedical Research and the Program of the Institute:

These results show that human (and other mammals) lung tumors ectopically express HCG in almost 80% of cases except SCLC, but that normal adult tissues do not, nor do tissues with hyperplastic and metaplastic disease. HCG is therefore highly correlated with lung cancer and a useful marker for neoplastic disease.

Normal human bronchial epithelial cells transformed in vitro by the v-Ha-ras oncogene yielded tumors which produced HCG. Since the in vitro cell progenitors of this tumor did not make large amounts of α- or β-HCG, HCG activation must occur in a step subsequent to V-Ha-ras transfection.

Since HCG was found to be a growth factor for bronchial epithelial cells, the possibility is raised that activation of HCG synthesis allows outgrowth of initiated bronchial cells, such as those transformed by v-Ha-ras. These studies have thus shown that HCG is a useful marker for human lung cancer and that it is highly possible that HCG has an important role in the pathogenesis of lung tumors.

Proposed Course:

The evident consistent appearance of β-HCG as a lung cancer marker inevitably provokes the question of whether it plays a contributing role in tumorigenesis.

We plan to extend the HCG growth stimulation finding by adding purified α or β subunits to cultures (in β- or α-depleted medium) of the lung tumor cell lines which we characterized as synthesizing only one of the subunits. Growth stimulation would imply that the tumor cells were still responsive to growth factor, but had circumvented normal exogenous, regulated supplies by synthesizing their own. Introducing the gene for the appropriate subunit as an expression vector should fulfill the same role. Introducing a vector expressing the reverse transcript (antisense RNA) blocks expression of the cognate gene in some systems. If such a construction of α- and/or β-HCG mRNA blocked growth stimulation, tumorigenesis in nude mice might also be blocked. A similar experiment would be possible with the ras-transformed bronchial epithelial cells. We also plan to examine HCG synthesis in additional tumors derived

from the ras-transfected bronchial cells to determine how typical HCG expression is and at what stage HCG is first expressed. HCG mRNA expression studies will be continued for the same reason.

Finally, it is of interest to assay transient HCG expression transfection with activated ras oncogenes to determine whether HCG is an early, but perhaps transient event as is the case for some cellular proto-oncogenes induced by oncogenes.

Publications

Yoakum, G. H., Lechner, J. F., Gabrielson, E., Korba, B. E., Malan-Shibley, L., Willey, J. C., Valerio, M. G., Shamsuddin, A., Trump, B. F. and Harris, C. C.: Transformation of human bronchial epithelial cells transfected by Harvey ras oncogenes. Science 227: 1174-1179, 1985.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01CP05428-01 LHC

PERIOD COVERED
October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Genomic Libraries from A1146, Human Bronchial Epithelial Cells and Fibroblasts

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Paul Amstad	Visiting Fellow	LHC	NCI
Others:	Curtis C. Harris	Chief	LHC	NCI
	Ainsley Weston	Visiting Fellow	LHC	NCI
	George Mark	Expert	LHC	NCI
	Hans Krokan	Guest Researcher	LHC	NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH
Laboratory of Human Carcinogenesis

SECTION
Carcinogen Macromolecular Interaction Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.0	1.0	

CHECK APPROPRIATE BOX(ES)
☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*
Genomic libraries are necessary tools to isolate and characterize known or unknown genes. In studying lung carcinogenesis, we attempt to investigate the role of oncogenes and genes which are involved in the regulation of differentiation and in the process of transformation. We decided to construct genomic libraries from a transformed human lung carcinoma cell line A1146 and from normal human bronchial epithelial (NHBE) cells. In addition, a genomic library from human normal fibroblasts was constructed too. To assess the completeness of the libraries, both the A1146 and the NHBE libraries were screened for the presence of alpha and beta human chorionic gonadotropin genes by plaque hybridization.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

Paul Amstad	Visiting Fellow	LHC	NCI
Curtis C. Harris	Chief	LHC	NCI
Ainsley Weston	Visiting Fellow	LHC	NCI
George Mark	Expert	LHC	NCI
Hans Krokan	Guest Researcher	LHC	NCI

Objectives:

Genomic libraries are used to identify and characterize human genes involved in transformation and differentiation of NHBE cells, the main target cell for lung cancer.

Methods Employed:

High molecular weight DNA was isolated with an average size bigger than 50 kb. The DNA was partially digested with the restriction enzyme Sau3A to an extent that most of the DNA molecules are between 9 and 23 kb in size. The partially digested DNA was then located on a 10-40% sucrose density gradient for sizing. Fractions of the gradient were then analyzed on a 0.4% agarose gel. Fractions containing DNA molecules between 9 and 23 kb in length were pooled and ethanol precipitated. The insert DNA was then cloned into the BamHI site of $EMBL_3$ or $EMBL_4$ cloning phages. The recombinant phage DNA was subsequently packaged in an in vitro packaging system and assayed in an NH539 indicator bacteria which allow only recombinant phages to grow and to form plaques. The plating efficiency was usually around 5×10^5 pfu/μg insert. In order to screen the libraries, 5×10^5 pfu of the amplified library were plated on 10 x 15 1 cm petri dishes containing bottom agar and plaque lifts were made. The nitrocellulose filters were then probed with P^{32}-labeled fragments of α and β human chorion gonadotropin genes. Positive plaques were isolated and subjected to two rounds of plaque purification.

Major Findings:

Genomic libraries from A1146, NHBE and human fibroblastic cells have been established. They were screened for the presence of α and β HCG genes, which are differentially expressed in normal and transformed cells.

Significance to Biomedical Research and the Program of the Institute:

The use of genomic libraries is a prerequisite to study carcinogenesis on a molecular basis.

Proposed Course:

Currently, attempts are being made to isolate a genomic clone of the human c-pks, a raf-related gene isolated from an expression library. In the future, both the A1146 and the NHBE libraries will be used to isolate genes involved in differentiation by screening them with substracted cDNA clones or substracted cDNA probes.

Publications

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01CP05429-01 LHC

PERIOD COVERED
October 1, 1984 to September 30, 1985

TITLE OF PROJECT (*80 characters or less. Title must fit on one line between the borders.*)
Use of Retroviral Shuttle Vector for Infection of Oncogenes into Human Cells

PRINCIPAL INVESTIGATOR (*List other professional personnel below the Principal Investigator.*) (*Name, title, laboratory, and institute affiliation*)

PI:	Paul Amstad	Visiting Fellow	LHC	NCI
Others:	Curtis C. Harris	Chief	LHC	NCI
	George Mark	Expert	LHC	NCI

COOPERATING UNITS (*if any*)

LAB/BRANCH
Laboratory of Human Carcinogenesis

SECTION
Carcinogen Macromolecular Interaction Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.0	1.0	

CHECK APPROPRIATE BOX(ES)
☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (*Use standard unreduced type. Do not exceed the space provided.*)

The introduction of foreign genes into a retroviral shuttle vector offers the opportunity of infecting a variety of human cell types at high frequency. The coding region of the v-Ha-ras oncogene was cloned into the murine retroviral shuttle vector pZip-neo SV(X). Plasmid DNA of bacterial clones containing the Ha-ras coding region was isolated and transfected into NIH 3T3 cells by the calcium phosphate precipitation method. One out of six clones transfected was biologically active in induction of transformed foci.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

Paul Amstad	Visiting Fellow	LHC	NCI
Curtis C. Harris	Chief	LHC	NCI
George Mark	Expert	LHC	NCI

Objectives:

The introduction of foreign genes into a retroviral shuttle vector offers the possibility of infecting a variety of mammalian cells, including human, at a very high frequency. We focus on studying transformation of normal human bronchial epithelial (NHBE) cells and their regulation of differentiation by using the retroviral shuttle vector as a means of effectively transferring genes into human cells.

Methods Employed:

The 1.8 kb Bgl II fragment of clone H1 containing the structural gene of v-Ha-ras was isolated, recessed with Bal 31 to between 0.9 and 1.2 kb in length and then cloned into the unique BamHI site of the Zip-neo SV(X) shuttle vector. Bacterial clones of HB101 containing the recombinant plasmid were identified by colony hybridization using the 1.8 kb Bgl II ras fragment as a probe. Fourteen v-Ha-ras/Zip recombinants were isolated. Eight of those contained the insert in frame orientation relative to the transcription start site within the viral LTR. Six of the eight clones with the desired orientation were tested for biological activity by transfection into NIH 3T3 cells. One clone was found to be positive in the NIH 3T3 transfection assay.

Major Findings:

The structural gene of v-Ha-ras was cloned into the retroviral shuttle vector Zip-neo SV(X). Out of six clones assayed on NIH 3T3, one was shown to be positive in inducing transformed foci.

Significance to Biomedical Research and the Program of the Institute:

Besides the classical ways of introducing genes into mammalian cells (calcium phosphate precipitation or protoplast fusion method), the retroviral shuttle vector offers the advantage of a very high frequency of transfection of stable integration of the foreign gene of one copy per cell and the recovery of sequences introduced into the genome of the mammalian cell in a type of marker rescue experiment.

Proposed Course:

We are in the process of making a recombinant virus by transfecting the recombinant plasmid into cells containing integrated copies of a packaging

deficient M-Mulv provirus providing all the functions necessary for the encapsidation of the recombinant retrovirus. By infecting a v-Ha-ras/Zip recombinant retrovirus containing the v-Ha-ras insert in apposite orientation with respect to the direction of transcription into v-Ha-ras transformed NHBE cells, it should be possible to hybrid arrest the transcription of the in-frame v-Ha-ras stably integrated in NHBE cells (TBE-1 cells).

Publications

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05430-01 LHC

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT (*80 characters or less. Title must fit on one line between the borders.*)

Substracted cDNA Cloning and Substracted cDNA Probes

PRINCIPAL INVESTIGATOR (*List other professional personnel below the Principal Investigator.*) (*Name, title, laboratory, and institute affiliation*)

PI:	Paul Amstad	Visiting Fellow	LHC	NCI
Others:	Curtis C. Harris	Chief	LHC	NCI

COOPERATING UNITS (*if any*)

LAB/BRANCH

Laboratory of Human Carcinogenesis

SECTION

Carcinogen Macromolecular Interaction Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.5	0.5	

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK (*Use standard unreduced type. Do not exceed the space provided.*)

We are trying to molecularly characterize transformed cells versus normal cells on an expression level. One approach to achieve this goal is to make a substracted cDNA library which should contain only genes expressed in one but not in the other cell type. Another possibility is to make a P32-labeled substracted cDNA probe which can be used to screen a genomic library.

PHS 6040 (Rev. 1/84)

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

Paul Amstad	Visiting Fellow	LHC	NCI
Curtis C. Harris	Chief	LHC	NCI

Objectives:

Isolation and characterization of genes which are differentially expressed in a transformed cell compared to a normal one.

Methods Employed:

Poly A^+ RNA from A1146 and normal human bronchial epithelial (NHBE) cells are isolated by selecting three times over oligo (dT) cellulose. The mRNA from one cell type (A1146 or NHBE) was reverse transcribed into cDNA. The newly synthesized cDNA was then hybridized to a 20-fold excess of the competitor RNA from the other cell type. The remaining sscDNA was excluded from the bulk of RNA/DNA hybrids and RNA by passing it through a hydroxyapatite column using 0.12 M phosphate as eluant which elutes only sscDNA, whereas RNA and DNA/RNA hybrids are returned. After synthesis of the second DNA strand the DNA gets C-tailed and cloned into a G-tailed pBR322 plasmid. The substracted cDNA containing plasmids are then transformed into E. coli HB101.

Instead of cloning the substracted cDNA into pBR322, it is possible to run the reverse transcription in the presence of one P32-labeled triphosphonucleotide. The P32-labeled sscDNA is then hybridized to an excess of competitors in RNA and selected on hydroxylapatite as described before. The substracted P32-labeled sscDNA can be used as a probe to screen either a cDNA library or a genomic library to isolate differentially expressed genes.

Significance to Biomedical Research and the Program of the Institute:

Subtracted cDNA libraries or subtracted probes are widely used to isolate genes which are differentially expressed in the process of differentiation or transformation.

Proposed Course:

After having established a substracted cDNA library and/or substracted probes, both the A1146 and the NHBE gene libraries will be screened for genes which are uniquely expressed in one or the other phenotype.

Publications

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05431-01 LHC

PERIOD COVERED
October 1, 1984 to September 30, 1985

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)
Transfection of Oncogenes into Human Bronchial Epithelial Cells (NHBE)

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	Paul Amstad	Visiting Fellow	LHC	NCI
Others:	Curtis C. Harris	Chief	LHC	NCI
	George Yoakum	Senior Staff Fellow	LHC	NCI
	Brenda Gerwin	Research Chemist	LHC	NCI

COOPERATING UNITS (if any)

LAB/BRANCH
Laboratory of Human Carcinogenesis

SECTION
Carcinogen Macromolecular Interaction Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.0	1.0	

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

NHBE cells were transfected with a variety of different oncogenes: raf, v-Ha-ras, a combination of raf and v-myc on the same plasmid and the translocated c-myc frame the CA46 Burkitt's lymphoma (BL) cell line. The transfected cells were then selected for resistance to inducers of differentiation by treating them with blood derived serum (BDS) or TBA. The different oncogenes have also been tested on NIH 3T3 cells for their capability to induce transformation. Both assays showed the CA46 translocated c-myc gene as the most effective oncogene not only in inducing resistance to differentiation in normal human bronchial epithelial (NHBE) cells, but also in inducing transformation in NIH 3T3 cells. The translocated c-myc gene has been further analyzed by testing deletion mutants of the original clone for resistance to differentiation and induction of transformation in both biological assay systems.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

Paul Amstad	Visiting Fellow	LHC	NCI
Curtis C. Harris	Chief	LHC	NCI
George H. Yoakum	Senior Staff Fellow	LHC	NCI
Brenda Gerwin	Research Chemist	LHC	NCI

Objectives:

To study the role of oncogenes especially c-myc in transformation and in the regulation of differentiation in NHBE cells and NIH 3T3 cells.

Methods Employed:

NHBE cells have been tranfected with plasmid constructs containing oncogenes by the protoplast fusion method. Three days after the fusion the transfected cells were subcultured to 500,000 cells per dish and one day later selected with 2% BDS on 10 mM TPA for about 3 weeks. After one to two weeks, the appearance of clones resistant to inducers of differentiation were observed. Three weeks after the initial treatment, the dishes were fixed and stained with crystol violet. Colonies were then counted. The selection was done in LHC-8 medium, a defined growth medium which is devoid of epinephrine and retinoic acid which antagonize BDS and TPA in inducing differentiation.

The oncogenes were transfected into NIH 3T3 by the calcium phosphate precipitation method. Deletion mutants of the original CA46 c-myc clone were made by removing either the IgH part or the switch region or the second and third exon of c-myc by standard recombinant DNA technique.

Major Findings:

Among the oncogenes tested on HNBE for induction to resistance of differentiation, the translocated c-myc from CA46 cells showed the strongest response. The NIH 3T3 assay for transformation yielded the same result with a very high induction of transformation by the CA46 c-myc. Clones of NHBE cells resistant to inducers of differentiation like BDS and TPA showed an increased growth potential but did not become immortal; neither was their morphology altered.

Significance to Biomedical Research and the Program of the Institute:

Sensitivity to inducers of differentiation like BDS, TPA or βTGF is a major characteristic of NHBE cells. It has been shown in our laboratory that the transfection and the stable integration of v-Ha-ras into NHBE leads not only to resistance of those transfected cells to inducers of differentiation but also to morphological alteration and finally to transformation. The search for other

oncogenes which may have similar effects on the process of transformation became obvious. The human c-myc gene has not yet been reported to be a fully transforming gene. Rather, it acts as a stimulator of cell proliferation in a cell cycle dependent fashion. It has also been shown that the myc oncogene is capable to induce the expression of another oncogene which finally leads to transformation. The NIH 3T3 assay clearly showed that a translocated c-myc gene can induce transformation by itself.

Proposed Course:

We are conducting experiments which should clarify which part of the CA46 c-myc clone is responsible for transformation in NIH 3T3 cells. β-TGF as the main differentiation inducing component of BDS is being used instead of BDS for selection of transfected NHBE cells. In a later stage of the experiment mRNA from transfected cells will be isolated and analyzed in a SV protection assay to prove that the transfected CA46 c-myc gene is present in the cells and expressed.

Publications

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05432-01 LHC

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

The Biological Activity of Fecapentaene-12 in Human Tissues Cells

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	Simon M. Plummer	Visiting Fellow	LHC	NCI
Others:	Curtis C. Harris	Chief	LHC	NCI
	Tsyochi Kakefuda	Medical Officer	LMC	NCI
	Kaija Linnainmaa	Visiting Fellow	LHC	NCI
	Hiroshi Imai	Visiting Fellow	LMC	NCI

COOPERATING UNITS (if any)

Microbiological Associates, Inc., Bethesda, MD (R. Curren, L.L. Yang, D. Putman); Dept. Toxicology, Karolinska Institute, Sweden (R. Grafstrom); Dept. Pathology, University of Maryland, Baltimore, MD (A.K.M. Shamsuddin)

LAB/BRANCH

Laboratory of Human Carcinogenesis

SECTION

Biochemical Epidemiology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
5.0	2.0	3.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Fecapentaene-12 (fec-12), a possible initiating agent in colon cancer, is cytotoxic and mutagenic in human fibroblasts. DNA repair deficient fibroblasts are more sensitive than normal fibroblasts to both these effects, which are dose dependent. Further studies with human fibroblasts have shown that fec-12 is a potent inducer of single-strand breaks (SSB) in DNA. Accumulation of these breaks as a result of inhibition of the polymerase component of the excision repair mechanism suggests that their formation may be mediated in part by DNA repair mechanisms. Autoradiographic techniques have shown that fec-12 is capable of inducing unscheduled DNA synthesis (UDS) in human fibroblasts. These results indicate that fec-12 is genotoxic and mutagenic to human cells. Further support for the hypothesis that fec-12 is an initiating agent in colon cancer comes from the finding that this compound is an inducer of transformation in murine Balb 3T3 cells. Plasmid assays investigating the nature of fec-12-DNA interaction have shown evidence of interstrand DNA cross-links. Results from electron microscopic studies support these findings and also suggest that fec-12 can directly cause DNA strand breaks.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

Simon Plummer	Visiting Fellow	LHC	NCI
Curtis Harris	Chief	LHC	NCI
Tsyochi Kakefuda	Medical Officer	LMC	NCI
Kaija Linnainmaa	Visiting Fellow	LHC	NCI
Hiroshi Imai	Visiting Fellow	LMC	NCI

Objectives:

To assess the possible importance of fec-12 in the etiology of human colon cancer by studying (1) genotoxicity and mutagenicity in human cells and tissues; (2) transforming ability in a mammalian cell assay; (3) molecular mechanism(s) of fec-12-induced DNA damage by in vitro assays.

Methods Employed:

Monolayer cultures of human fibroblasts have been used to study the cytotoxicity and mutagenicity of fec-12 in human cells. Fec-12-induced single-stranded breaks (SSB) and unscheduled DNA synthesis have also been measured in this cell culture system by alkaline elution and autoradiography, respectively. Techniques developed in this laboratory for the culture of explants of human colon have been used to study the cytotoxicity of fec-12 in the probable target tissue. Biochemical and morphological markers of cytotoxicity and genotoxicity have been studied by measuring radiolabeled precursor incorporation and doing light and electron microscopy in tissue sections, respectively. A plasmid DNA cross-linking assay has been developed to study the molecular mechanism(s) involved in fec-12-induced DNA damage. Electron microscopy has also been employed in these studies.

Major Findings:

Fec-12 is cytotoxic and mutagenic in human fibroblasts. DNA repair deficient fibroblasts are more sensitive than normal fibroblasts to both these effects. Fec-12 also causes single-strand breaks and induces unscheduled DNA synthesis in human fibroblasts. Fec-12 is capable of inducing a significant increase in the transformation frequency of murine Balb 3T3 cells in culture. These results provide evidence to support the hypothesis that fec-12 may be an initiating agent in colon cancer. In vitro studies into the mechanism of fec-12-DNA damage have established that this compound causes interstrand DNA cross-linking and DNA fragmentation.

Significance to Biomedical Research and the Program of the Institute:

Thirty percent of all North Americans have mutagenic feces and 5% have a high level of mutagenic activity. The mutagenic activity of organic solvent extracts of human feces has been shown to be due almost entirely to a single compound or group of compounds called fecapentaene(s). Increased fecapentaene levels have been shown to correlate with the type of diet associated with an increased risk of colon cancer. It is therefore possible that fecapentaene may be an important etiological agent in colon cancer. Our studies have shown that fec-12 is mutagenic

in human cells which provides suggestive evidence that this chemical may also be carcinogenic. Further support for this hypothesis comes from the finding that fec-12 causes transformation in mammalian cells.

Studying the mechanism of action of this chemical may provide new clues to mechanisms involved in initiation of colon cancer. We have established that fec-12 is capable of causing DNA-stranded breaks in human cells and cross-linking as well as stranded breaks in plasmid DNA.

Proposed Course:

Since results from plasmid assays suggest that fec-12 reacts covalently with DNA to form adducts we have decided to isolate and characterize these adducts. This will involve the isolation of adducts by DNA digestion and HPLC which will then be characterized by synchronous fluorescence spectroscopy. It is hoped to raise antibodies to these adducts in order to develop immunoassays for their measurement. These techniques will be used to try to measure adducts in human colon DNA extracted from biopsy and immediate autopsy specimens.

Evidence from plasmid cross-linking assays and electron microscopic studies suggest that fec-12 can form interstrand DNA cross-links. It is known that bifunctional alkylating agents, capable of forming DNA cross-links, induce sister chromatid exchanges (SCE) with high efficiency on a molar basis when compared to noncross-linking agents. It is intended to investigate the ability of fec-12 to induce SCE in human fibroblasts. The formation of DNA-DNA cross-links in human fibroblasts will also be measured by alkaline elution.

Molecular changes involved in the induction of mutation by fec-12 in bacterial cells will be studied by using a plasmid-mediated mutation assay. With this assay it will be possible to analyze specific changes in DNA sequence as a result of fec-12-DNA damage.

The pathological effects of fec-12 in cultured human colonic explants and epithelial cells will be investigated.

Publications:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05433-01 LHC

PERIOD COVERED
October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Synchronous Fluorescence Scanning Detection of Aflatoxin Adducts

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Vincent L. Wilson	Senior Staff Fellow	LHC	NCI
Others:	Dean Mann	Section Chief	LHC	NCI
	Curtis C. Harris	Chief	LHC	NCI

COOPERATING UNITS *(if any)* Boston Univ., School of Public Health, Boston, MA (J.D. Groopman); Dept. Pathology, Univ., of MD, School of Medicine, Baltimore, Maryland (A.K.M. Shamsuddin); Dept. of Immunology, Cancer Institute, Chinese Academy of Medical Science, Beijing, PRC (Sun. Tsung-tang)

LAB/BRANCH
Laboratory of Human Carcinogenesis

SECTION
Biochemical Epidemiology Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.6	0.3	0.3

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Synchronous fluorescence scanning and computer linkages for data analysis have been used to characterize the fluorescent properties, limits of detection, contour maps and 3-dimensional images of a series of aflatoxins, their metabolites, and DNA adducts. The power of these fluorescent techniques has been shown by the demonstration that the number of components within a sample can be delineated using fourth derivative computer analysis of 3-dimensional synchronous fluorescence scanning data. Simple methylation of the 9 position of the guanine residue in AFB1-N7-guanine not only did not increase fluorescence, as has been proposed, but also caused a shift in the optimum delta lambda. Thus, these techniques may provide valuable structural determinations of fluorescent carcinogens and DNA adducts.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on This Project:

Vincent L. Wilson	Senior Staff Fellow	LHC	NCI
Dean Mann	Section Chief	LHC	NCI
Curtis C. Harris	Chief	LHC	NCI

Objectives:

To develop fluorescent techniques for detection of minor levels of aflatoxin adducts in DNA. To determine if liver alfatoxin DNA adduct levels correlate with the incidence of hepatic cancer in susceptible populations. To establish the usefulness of the analysis of aflatoxin DNA adduct levels to the study of the relationship of carcinogen activation and binding to human cancer.

Methods Employed:

DNA treated with AFB_1 in the presence of a microsomal enzyme activating system, liver DNA from AFB_1 treated rats, and human liver DNA from unknown environmental exposure to AFB_1, will be analyzed by synchronous fluorescence scanning on 650-40 Perkin-Elmer fluorescence spectrophotometer linked to the Perkin-Elmer 3600 data station. Further data manipulation will be on the NIH Dec-10 computer system. DNA of known AFB_1 exposure will also be assessed for the levels and pattern of AFB_1-DNA adducts by HPLC.

Major Findings:

Detection and characterization of the fluorescent properties of several aflatoxins, their metabolites, the major DNA adduct AFB_1-N^7-guanine, and DNA modified with AFB_1, has been performed. The sophistication of the available fluorescence instrumentation has enabled the sensitive detection and fingerprint analysis of these compounds by synchronous fluorescence scanning (SFS) spectrophotometry. The limit of concentration detectable by SFS for each of these aflatoxins was determined. Although AFB_1-N^7-guanine, the major DNA alkylation product of aflatoxin B_1, was readily detectable by SFS, the conversion of this adduct to the ring-opened AFB_1-FAPY product dramatically reduced the detectability of DNA covalently bound aflatoxin. The optimum delta lambda for each compound was also determined, thus providing the spectral characteristics that distinguish each of these hydrocarbons from one another.

The ability to detect and differentiate fluorescent products in biological fluids and DNA may be feasible due to the adaptation of these techniques to 3-dimensional analysis and the production of contour maps. Data collection for 3-dimensional analysis requires only taking a series of SFS scans over a range of delta lambdas. Contour maps and 3-dimensional images are extremely specific to a given compound, much like a fingerprint. Computer assisted analysis of 3-dimensional data provided 4th derivative determinations which demonstrated the presence of two components in a standard mixture of AFB_1 and

AFM_1. SFS computer assisted analysis of biological samples taken where environmental exposure may have occurred, may, therefore, provide determinations of how many fluorescent components are present and possibly what some of them are.

The quantitativeness of AFB_1 adducts in DNA by SFS analysis has been demonstrated using in vivo treated rats for a model study. The liver DNA from AFB_1 treated rats was used to determine the level of AFB_1 bound to DNA. These values compared well with those determined by radiolabeled AFB_1 used in the dosing of the rats. However, the SFS analysis of numerous human liver DNA samples provided no significant levels of AFB_1 bound to DNA. Thus, the limit of detection for AFB_1-DNA adducts may not be low enough to analyze for environmental AFB_1 exposure.

Methylation of the N^9 position of the guanine residue in AFB_1-N^7-guanine had been proposed to enhance fluorescence. SFS analysis of AFB_1-N^7-Me-guanine demonstrated that not only did the optimum delta lambda increase, but the fluorescence of this adduct decreased as compared to the parent structure. Other adduct modifications such as glycosylation, will have to be tested to determine if the SFS detection of AFB, adducts can be enhanced.

Significance to Biomedical Research and the Program of the Institute:

Carcinogen-DNA adducts are pathological lesions which are considered to be important to the initiation and/or progression of carcinogenesis. Recently, both immunological and physical methods have been developed to measure adducts in macromolecules such as DNA isolated from carcinogen-exposed tissue and cells. The development of SFS techniques would not only compliment these other methods, but would also provide a highly selective analysis by the fingerprinting of known adducts. The results of such studies may enable the advancement of research in the epidermological relationship of environmental carcinogen exposure to cancer incidence.

Proposed Course:

Improvement in the SFS detection of aflatoxin adducts in biological DNA samples may enable epidemiological comparisons in cancer incidence and aflatoxin exposure. Two separate experimental routes will be used to improve AFB_1-N^7-guanine and AFB_1-DNA detection. Separation of AFB_1-DNA adducts from the bulk of DNA will be performed by HPLC or immunocolumn chromatography before SFS analysis, thus concentrating the fluorescent moieties prior to analysis. Alternatively, chemical derivatization of AFB_1-DNA adducts may enhance the fluorescent properties and SFS detectability of these compounds. Simple methylation of AFB_1-N^7-guanine did not improve the fluorescent character of this adduct, but other forms of modification need to be tested.

The capacity of the SFS 3-dimensional fingerprint analysis has not been tested. In the interest of developing a means of identifying selective fluorescent

components in unknown biological samples, a library of fluorescent data on a broad range of carcinogens and their DNA adducts will be collected. Each compound will have its own fingerprint contour map and 3-dimensional image. Thus, by programming the computer to compare a 3-dimensional image of an unknown sample with a search through a library of fluorescent fingerprint data, the component or components that make up the unknown may be identifiable.

Publications

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01CP05434-01 LHC

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

The Immunobiology of AIDS and AIDS-Related Diseases

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	Dean L. Mann	Medical Officer	LHC	NCI
Others:	William Blattner	Chief, Family Studies Section	EEB	NCI
	J. J. Goedert	Expert	EEB	NCI
	R. J. Bigger	Medical Officer	EEB	NCI
	Mark H. Green	Medical Officer	EEB	NCI
	Stanley H. Weiss	Medical Staff Fellow	EEB	NCI
	R. C. Gallo	Chief	LTCB	NCI
	Mika Popovic	Medical Officer	LTCB	NCI

COOPERATING UNITS (if any)

Uniformed Services, University of the Health Sciences, Bethesda, MD (M. Newman)

LAB/BRANCH

Laboratory of Human Carcinogenesis

SECTION

Biochemical Epidemiology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.0	0.5	0.5

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Acquired immunodeficiency syndrome (AIDS) is characterized by profound loss of the ability to respond to environmental antigens as well as the development of Kaposi's sarcoma. We have studied the percentages of T-cell subsets in patients with the disease and patients at risk for the disease. The at-risk population has been hemophiliacs and male homosexuals. Studies were conducted using lymphocytes from individuals in the Washington, DC area. Low T-helper cells (OKT-4-+) were found in homosexuals who had contacts with high-risk groups, individuals from the New York and San Francisco areas. Sexual practices of individuals at risk for the disease and those individuals with AIDS were investigated to see if there was a correlation with low helper T-cells. The number of sexual partners correlated with the lower numbers of OKT-4+ cells as did receptive anal intercourse. The HTLV-III retrovirus has been isolated from AIDS patients and individuals at risk for this disease. A positive correlation of low OKT-4+ cells was found with antibody to the HTLV retrovirus. There was also a correlation of the presence of this antibody with large numbers of homosexual partners as well as receptive anal intercourse. We investigated an in vitro model for the depletion of specific T-cell subsets using lymphocytes from patients with AIDS, male homosexuals and normal individuals. Depletion of OKT-4 cells in culture was present when the lymphocytes were stimulated with PHA and alpha interferon was present. The selective loss of OKT-4-positive cells in these individuals suggest that these cells bear the specific receptor for the HTLV-III retrovirus. We have identified the specific portion of the OKT-4 molecule which binds the HTLV-III retrovirus. In addition, the OKT-4 molecule seems be downregulated in expression with continued infection of cell lines with the HTLV-III retrovirus.

PHS 6040 (Rev. 1/84)

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

Dean L. Mann	Medical Officer	LHC	NCI
William Blattner	Chief, Family Studies Section	EEB	NCI
Robert Gallo	Chief	LTCB	NCI
Robert Bigger	Medical Officer	EEB	NCI
Stanley Weiss	Medical Staff Fellow	EEB	NCI
Mika Popovic	Medical Officer	LTCB	NCI

Objectives:

The objective of this investigation is to study the immunobiology of AIDS and AIDS-related diseases. These studies include investigation of the action of the HTLV retrovirus on cell depletion, i.e., cell death, the specific receptor site for the attachment of the HTLV-III retrovirus and the mechanism of destruction of these helper cells. The studies are directed also at the understanding of the nature of the determinant on the retroviral envelope which attaches to the OKT4 receptor and in addition, investigation of the immune response of individuals who are infected with this retrovirus who do not develop AIDS or AIDS-related diseases.

Methods Employed:

Monoclonal antibodies detecting the OKT-4 antigenic determinants as well as OKT8 were used to study lymphocytes from the patients with AIDS or the male homosexuals who are at risk for the development of AIDS. Peripheral lymphocytes were obtained from populations of these individuals cryopreserved until used. The cells were thawed, reacted with the monoclonal antibodies and a rabbit antimouse immunoglobulin added. After the appropriate periods of incubation, the cells were analyzed on the fluorescence-activated cell sorter. All cells showing a forward light-scatter pattern were analyzed. The percentages of the total population reacting with the monoclonal antibody was determined. Sera were obtained from these patients at the time that lymphocytes were being procured. This was tested in the enzyme-linked immunoassay (ELISA) for antibody activity to the HTLV-III retrovirus.Ratios of binding of the sera to the retrovirus that were five times greater than the binding to control plates were considered positive reactions for this retrovirus. Specific questionnaires inquiring into the life-styles and sexual practices of the individuals were formulated by the Environmental Epidemiology Branch of the NCI. The results of the data collected on these questionnaires were correlated with the numbers of OKT-4 helper cells and/or helper suppressor ratios by standard statistical techniques. Lymphocytes from these patients were placed in culture with PHA without PHA, PHA with acid labile or acid stabile alpha interferon.Controls were males who denied homosexual contact. Sera from these individuals were analyzed for antibodies HTLVIII. Five patients with AIDS and 15 male homosexuals were studied. After three days in culture, the cells were fed with tissue culture media containing the alpha interferon. After seven days, the total

numbers of OKT-4 and OKT-8 cells were determined using the fluorescence-activated cell sorters described above. The H9 cell line was found by Popovic et al. to be susceptible to infection with the HTLV retrovirus and to propagate this retrovirus in vitro. The cell line was used to study the receptor for the HTLV-III retrovirus. The isolated sucrosebanded retrovirus was incubated with the H9 cell line for various time periods. The cells were cooled, fixed and analyzed for the binding of a series of monoclonal antibodies detecting the different epitopes in the OKT-4 molecule.

Major Findings:

The studies on the T-cell subsets were carried in cooperation with the Environmental Epidemiology Branch of the NCI. Lymphocytes were obtained from selected populations as described above. Questionnaires were designed to attempt to correlate certain environmental factors as well as activities that may predispose to the disease. One hundred and eighty homosexual men representing patients from two physicians' offices in Washington, DC were enrolled in the study. Lymphocytes obtained from these individuals were studied for the presence of OKT-4 and OKT-8 determinants using the fluorescence-activated cell sorter described above. The data obtained from the laboratory analysis were correlated with the patient history of homosexual contacts with indivudals in known highrisk areas: New York City, San Francisco or Los Angeles. There was a positive correlation with decreased OKT-4-positive cells in those individuals who had homosexual contacts with individuals in the high risk areas. The population could be stratified into those who were at high risk (that is, had sexual contact with individuals in the high endemic areas); intermediate risk, those individuals who had homosexual contact with individuals in the Washington, DC areas who are considered in the high-risk group and low-risk individuals; and those individuals who had not had homosexual contact with the above two groups. There was a positive correlation of the total number of OKT-4 and decreased helper suppressor ratios in this population. An analysis of the subject population from the Washington, DC area as well as the New York area showed that another substantial risk factor in the development of depressed OKT-4-positive cells was anal intercourse. In addition, it was observed that increased numbers of sexual partners in this group was additive and that the above two activities combined gave lower helper T-cell numbers. Analysis of the data of survival OKT-4 lymphocytes from AIDS patients, the homosexual males and normal males showed that there was a selective decrease in the OKT-4 population after seven days culture with PHA in the presence of alpha interferon. PHA alone or alpha interferon did not appreciably affect survival in the populations who had antibody to HTLV-III. These results demonstrate that co-factors such as lymphocyte-stimulation in the presence of circulating alpha interferon may be significant in the development of the immunodeficiency seen in this patient population.
The specific receptor site for the HTLV III retrovirus has been identified on the OKT-4-positive cells. In the short-term exposure of the H9 cells with the HTLV-III, the epitopes on the OKT-4 molecule detected by OKT-4A, OKT-4D and OKT-4F disappeared, while the epitope detected by the OKT-4 antibody remained intact for

48 to 60 hours. All cell lines which had been infected with the HTLV-III retrovirus did not express the OKT-4 determinant. This was true of infection of cell lines with three different retroviral isolates which would differ considerably in the env portion of the retroviral DNA. The data suggest that this retrovirus, while having considerable genetic diversity, uses the same receptor in all instances.

Significance to Biomedical Research and the Program of the Institute:

The acquired immunodeficiency syndrome is a deadly disease that appears to be increasing in epidemic proportions. It is imperative that an understanding of the nature of the action of the retrovirus, which in turn causes the profound immunodeficiency, be understood so that appropriate therapeutic and preventive modalities can be undertaken. Our early epidemiologic studies suggested a transmissible agent for the acquired immunodeficiency syndrome. These studies documented a decrease in the subpopulation of T lymphocytes in male homosexuals and those at risk for the disease. In our early studies we demonstrated that this decrease in the OKT-4 population was seen in Denmark homosexuals who had sexual contact with individuals in New York. Studies of the Washington, DC "gay" population also demonstrated this same phenomenon. With the isolation of the HTLV-III retrovirus the presence of seropositivity to this virus correlated with the decrease in the OKT-4-positive cells. All of the cell lines infected with the HTLV-III retrovirus showed a loss of expression of the OKT-4 molecule. Our results demonstrate that this molecule is a specific receptor site for the retrovirus. The in vitro depletion of OKT-4-positive cells suggests a model to explain the disappearance of the OKT-4-positive cells in this disease. The risk factor of high numbers of sexual partners is probably due to the increased stimulation with the histocompatibility antigens. Our results suggest that this chronic antigen stimulation with histocompatibility antigens together with the HTLV retrovirus infection may be one of the causative factors in the depletion of the OKT-4-positive cells and thus the profound immunodeficiency seen in this disease.

Proposed Course:

This project represents the combined efforts of a number of investigators in both clinic and laboratory. Antibody studies will be performed using sera from patients with AIDS and those at risk for the disease. As our studies are approached in an epidemiologic fashion, we will be able to follow individuals who convert to seropositive. Those individuals will be studied to see if they develop protective antibodies for this retrovirus. If such antibodies are found or suggested, the sera will be used to isolate the specific proteins or portions of the peptides that are encoded for by the retrovirus. The specific T-cell receptor on the lymphocyte suggests a complementary receptor on the HTLV-III retrovirus. Studies are underway to determine the specific receptor site on the HTLV-III retrovirus envelope which is attached specifically to the OKT-4 receptor.

Publications

Bigger, R. J., Melbye, M., Ebbesen, P., Mann D. L., Goedert, J. J., Weinstock, R., Strong, D. M. and Blattner, W. A.: Low T lymphocyte ratios in homosexual men: Epidemiological evidence for a transmissible agent. J. Amer. Med. Assoc. 251: 1441-1446, 1984.

Goedert, J. J., Bigger, R. J., Winn, D. M., Mann, D. L., Gallo R. C., Sarangadharan, M. G., Weiss, S. H., Grossman, R. J., Bodner A. J., Strong, D. M. and Blattner, W. A.: Determinants of retrovirus (ACLB-III) antibody and immunodeficiency conditions in homosexual men. Lancet 29: 711-716, 1984.

Goedert, J. J., Bigger, R. J., Winn, D. M., Mann, D. L., Byar, D. P., Strong, D. M., Digioia, R. A., Grossman, R. J., Sanchez, W. C., Kase, R. G., Greene, M. H., Hoover R. N. and Blattner, W. A.. Decreased helper T-lymphocytes in homosexual men. I. Sexual contact with high incidence areas for the acquired immune deficiency syndrome. Amer. J. Epidemiol. (In Press)

Goedert, J. J., Bigger, R. J., Winn, D. M., Mann, D. L., Byar, D. P., Strong, D. M. Digioia, R. A., Grossman, R. J., Sanchez, W. C., Kase, R. G. Greene, M. H., Hoover, R. N. and Blattner, W. A.: Decreased helper T lymphocytes in homosexual men. II. Sexual practices. Amer. J. Epidemiol. (In Press)

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01CP05435-01 LHC

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Analysis of Hydrocarbon-DNA Adducts in Humans and Relation to Cancer Risk

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Ainsley Weston	Visiting Fellow	LHC	NCI
Others:	Glennwood Trivers	Biologist	LHC	NCI
	Dean L. Mann	Section Chief	LHC	NCI
	Curtis C. Harris	Chief	LHC	NCI

COOPERATING UNITS *(if any)*

University of Oulu, Oulu Finland (K. Vahakangas)

LAB/BRANCH

Laboratory of Human Carcinogenesis

SECTION

Biochemical Epidemiology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.0	1.0	0.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Benzo[a]pyrene-diol-epoxide-DNA (BPDE-DNA) adducts were prepared synthetically and subjected to analysis by spectrophotometry, spectrophotofluorimetry and ultra-sensitive-enzyme-linked-radioimmunoassay (USERIA). The extent of DNA modifcation was originally determined to be 1% (1 adduct in 100 nucleotides) using the method of Santella et al. (Biochemistry 16:3127, 1977). It was further determined that 1 adduct in 1.4 x 10 to the seventh power nucleotides could be detected using spectrophotofluorimetry (2) and 1 adduct in 2.8 x 10 to the seventh power nucleotides could be detected by USERIA (1). Both of these methods were subsequently applied to the analysis of human DNA samples that were obtained from individuals who were occupationally and/or deliberately (cigarette smoking) exposed to polycyclic aromatic hydrocarbons (PAHs) (1,2). Among a group of coke oven workers, the presence of hydrocarbon-DNA adducts was detected in 31 out of 41 (76%) individuals by spectrophotofluorimetry and 18 out of 27 (67%) by USERIA; among groups of roofers and foundry workers, the presence of hydrocarbon-DNA adducts was detected in 7 out of 28 (25%) and 7 out of 20 (35%) individuals from these respective groups by USERIA. The results further showed that the presence of hydrocarbon-DNA adducts was related to the tobacco smoking habit.

However, occupational and other exposures to complex mixtures of PAHs are most usual. Correspondingly complex fluorescence spectra are frequently encountered and it may be that anti-BPDE-DNA antibodies cross-react with related hydrocarbon-DNA adducts. In order to adequately monitor human populations for exposure to PAHs and other related genotoxic chemicals, it will be necessary to design accurate methods for dosimetry based upon those already used to detect the presence of BPDE-DNA adducts in human samples.

PHS 6040 (Rev. 1/84)

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

A. Weston	Visiting Fellow	LHC	NCI
G. Trivers		LHC	NCI
D. Mann	Section Chief	LHC	NCI
C. Harris	Chief	LHC	NCI

Objectives:

1) To identify and characterize a range of hydrocarbon-DNA adducts that may arise as a result of exposure to cigarette smoke condensate. This will be achieved primarily through the development and use of both analytical and preparative HPLC, fluorescence spectroscopy and USERIA.

2) To prepare and examine hydrocarbon-haemoglobin adducts and develop a fluorimetric assay that will facilitate monitoring of human exposure to PAHs. This will include treatment of rodents in vivo with model compounds and mixtures of PAHs.

Methods Employed:

Synthetic DNA-adducts have hitherto been prepared from BPDE, chrysene-1,2-diol 3,4-oxide (CRDE) and benz[a]anthracene-8,9-diol 10,11-oxide (BADE). Briefly, these ultimately reactive forms were dissolved in DMSO and mixed with aqueous solutions of DNA in the ratio 1:10 (w/w). The extent of DNA modification using this method was approximately 1.0% for BPDE and 0.1 - 0.2% for CRDE and BADE when determined by UV spectroscopy. Characterization of BPDE-DNA adducts and products of acid hydrolysis (NHCl, 90°C, 3 hr) has been completed (2); this analysis included determination of the fluorescence excitation and emission spectra, fluorescence synchronous spectra and limits of detection using a Perkin Elmer 650-40 spectrophotofluorimeter in conjuction with a Perkin Elmer 3600 data station. These methods will be similarly applied to the CRDE-DNA and BADE-DNA adducts that have been prepared. These two hydrocarbon derivatives were deliberately chosen because the parent hydrocarbons are more abundant environmental contaminants than B[a]P and because it would be expected that their fluorescence characteristics are similar since their conjugated aromatic ring structures are identical to phenanthrene (scheme I). HPLC, using a reverse-phase C-18 ODS column (4.6 x 250 mm) that was eluted with a linear gradient (30-40%) of methanol in water, was used to separate the major products that were formed when the CRDE-DNA and BADE-DNA adducts were hydrolysed.

Major Findings:

It was found that a maximum synchronous fluorescence signal for BPDE-DNA adducts and BP-tetrols was obtained at a wavelength difference delta, lambda of 34 nm. Using this protocol, it was possible to detect as little as 5-20 fmol/ml of the pyrene chromophore. The levels of detection for the CRDE-DNA and BADE-DNA adducts have not yet been determined.

The three-dimentional fluorescence spectra of the BADE-DNA and CRDE-DNA adducts proved to be similar but they are not identical. Contour mapping analysis of the spectra should more clearly show any apparent variation in these spectra; however, the major peak in the synchronous fluorescence spectrum (Δλ 56 nm) appeared at 300/356 for both of these adducts. A similar comparison will also be made between B[a]PDE-DNA and B[e]PDE-DNA adducts since they both have a pyrene-like aromatic nucleus.

It has been possible to separate the major hydrolysis product of CRDE-DNA from that of BADE-DNA (presumably BA-tetrol and chrysene-tetrol) by HPLC and SFS analysis.

Although it was not possible, using the present HPLC system, to separate BA-tetrol from BP-tetrol, it was possible to detect the presence of both the phenanthrene chromophore and the pyrene chromophore in the mixture.

Significance to Biomedical Research and the Program of the Institute:

Hydrocarbon-DNA adducts are likely to arise in humans who are exposed through tobacco smoking or who are occupationally or otherwise exposed to carcinogenic PAHs. Identification and quantitation of carcinogen-DNA adducts is relevant to epidemiological studies since a variety of factors, in addition to gross exposure to environmental contaminants, influence the formation and persistence of carcinogen-DNA adducts. The factors include metabolism of chemicals to reactive and inactive forms, the types of hydrocarbon-DNA adducts formed and mechanisms of DNA repair. Dosimetry of carcinogen exposure is essential to an understanding of the role of carcinogen-DNA adducts and their persistence in the process of human carcinogenesis and sensitive methods for the detection and identification of carcinogen-DNA adducts will facilitate dosimetry of carcinogen exposure in human populations.

Since these factors (total exposure, metabolism and DNA repair) all contribute to the total extent of DNA damage, then genotoxicity may be most accurately assessed by direct determination of the presence and levels of carcinogen-DNA adducts and/or levels of reactive metabolites formed, which might be detected as carcinogen protein adducts. Techniques such as those described here might also be applied to monitoring exposure of individuals to carcinogens among populations with a relatively high cancer risk.

Proposed Course:

A range of hydrocarbon-DNA adducts will be prepared, either synthetically by reacting hydrocarbon derivatives with DNA in vitro or metabolically by incubating the parent hydrocarbons, or known metabolic intermediates, with rat liver microsomes in the presence of DNA. Chromatographic, spectrophotofluorimetric and immunologic methods will be used to characterize these adducts and will subsequently be applied to the analysis of samples of human DNA.

Publications

Vahakangas, K., Haugen, A. and Harris, C. C.: An applied synchronous fluorescence spectrophotometric assay to study benzo[a]pyrene-diol-epoxide-DNA adducts. Carcinogenesis (In Press).

ANNUAL REPORT OF

THE LABORATORY OF MOLECULAR CARCINOGENESIS

NATIONAL CANCER INSTITUTE

October 1, 1984 to September 30, 1985

The Laboratory of Molecular Carcinogenesis (LMC) plans, develops, and conducts a research program designed to (1) clarify the molecular biology of carcinogenesis; (2) elucidate the fundamental nature of the interaction of carcinogenic agents, especially chemical, with biological systems in the induction of cancer; (3) identify those environmental and endogenous factors which relate to and modify the carcinogenic process; and (4) clarify the metabolic regulatory processes which are related to carcinogenesis.

The goal of the Laboratory of Molecular Carcinogenesis is to understand the molecular basis of carcinogenesis with the view toward identifying susceptible populations and preventing human cancer. The research progam is designed to understand the molecular basis by which carcinogenic agents cause malignant transformation, and to identify and characterize those exogenous and endogenous factors involved in carcinogenesis. The Laboratory seeks to clarify the metabolic interaction of exogenous and endogenous agents in the living organism at the molecular, cellular and organism levels and seeks to understand the consequences of these interactions in terms of cell regulation and carcinogenesis. The processes are studied in biological preparations and cells from experimental animals and humans.

The course of the Laboratory research program has been markedly affected by the powerful new techniques of molecular biology and immunology. Several of our staff are highly experienced in DNA recombinant and related techniques, protein chemistry, and hybridoma technology. The power and precision of these techniques have had a highly positive influence on the progress of many of the projects of the Laboratory.

Metabolic Control Section - Studies (1) the metabolic activation and detoxification of the polycyclic hydrocarbons (PCH) and other carcinogens and drugs and the relationship of this metabolism to individual sensitivity and susceptibility to carcinogenesis; (2) regulation, and structure of the genes for the enzymatic system primarily responsible for the metabolic activation and detoxification of PCH and other chemical carcinogens.

This section studies the molecular events of malignant transformation induced by chemical carcinogens, mainly those of the PCH class. The aim is to understand the enzymatic conversion of carcinogens to either detoxified forms, or to active carcinogenic forms. Higher organisms have systems for the detoxification and elimination of foreign chemical compounds, including carcinogens. These systems primarily involve microsomal cytochrome P-450 mixed-function oxygenases, but also include epoxide hydratase and conjugating enzymes. The vast majority of foreign compounds are processed by these enzyme systems. The mixed-function oxygenases are influenced by a variety of environmental factors such as drugs, pesticides and carcinogens, and are influenced by the nutritional

and hormonal state of the animal. The age, sex and genetic makeup also determine enzyme activity. Work in this Laboratory provided the key studies which showed that this enzyme system was responsible for the activation of PCH procarcinogens to their ultimate carcinogenic forms. A primary goal is to define the enzymatic mechanism by which polycyclic hydrocarbons are activated either to carcinogenic forms or to detoxified products. As these enzymes are characterized and as sensitive methods are developed for their assay, it may be possible to characterize an individual's enzymatic makeup with respect to carcinogen metabolism and to understand the relationship between this metabolism and individual susceptibility to PCH-induced carcinogenesis.

The approach is to identify and fully characterize the enzymes responsible for carcinogen activation and metabolism. In addition, we seek to understand the molecular biology and regulation of this system both at the genetic and epigenetic levels. We plan to assess the types and amounts of these enzymes in human populations using molecular biological, immunological, and metabolic approaches. We will carry out multileveled investigations of the carcinogen metabolizing enzyme systems continuing our use of HPLC to study carcinogen metabolites, using monoclonal antibodies (MAb) and enzyme inhibitors to study the properties of the enzymes and using recombinant DNA and other molecular biological techniques to study the structure and regulation of the genes for the enzymes of carcinogen-metabolizing systems.

Xenobiotics such as drugs and carcinogens, as well as endobiotics such as steroids and fatty acids, are metabolized by the mixed function oxidase systems. Cytochrome P-450 is the key component of mixed function oxidases and the type and quantity of specific forms of cytochrome P-450 determine the disposition of a particular substrate. Monoclonal antibodies (MAbs) are specific probes for particular isoenzymes. Myeloma cells were hybridized with spleen cells of mice immunized with purified human placenta mitochondrial cytrochrome P-450. We obtained 25 independent hybridomas producing MAbs to the human placenta mitochondrial cytochrome P-450. Placenta microsomal and mitochondrial cytochromes P-450 play important roles in steroid metabolism. In addition 3-methylcholanthrene inducible cytochromes P-450 of rats are also inducible in human placenta by smoking. We have measured this induction with MAbs.

The multiplicity of cytochromes P-450 was examined with monoclonal antibodies (MAbs) to 3-methylcholanthrene (MC)-induced rat liver cytochrome P-450. A semiquantitative, direct radioimmunoassay (RIA) has been developed to measure cytochrome P-450 in the microsomes from various tissues in animals that are untreated, or treated with MC. The amounts of cytochrome P-450 in different tissues and species, including human samples such as placentas and lymphocytes, were examined by competitive RIA. Individual differences have been observed by this method, which is more reliable than measurements of enzyme activity. Placentas from women who smoked cigarettes contained greater amounts of cytochrome P-450 with the MAb-specific epitope than placentas from nonsmokers. The amount of MAb-specific cytochrome P-450 in human peripheral lymphocytes increased after treatment with benz(a)anthracene. In a second study interindividual differences were found in a small group of samples. RIAs with multiple MAbs have also been used to detect epitope-specific cytochromes P-450 in animal livers, with the goal of classifying various tissues with respect to MAb-specific cytochromes P-450. Much higher levels of cytochrome P-450 recognized by MAb 1-7-1 were observed in MC-treated rats and C57Bl/6 mice

than in untreated rats, MC-treated DBA/2 mice and guinea pigs. These analyses provide an approach to the study of cytochrome P-450 multiplicity that is complementary to enzymatic and structural studies. RIA methods will aid in defining the epitope-specific cytochrome P-450 content in different tissues, species, and strains of laboratory animals, and in understanding the diversity of the cytochromes P-450 and their role in individual susceptibility to carcinogenesis.

The cytochromes P-450 metabolize a variety of drugs and carcinogens. The multiple forms of this enzyme display unique yet broad, substrate and reaction specificity. The focus of this project is the identification, characterization, and elucidation of structure-function relationships of these isozymes. Monoclonal antibodies (MAbs) to specific cytochromes P-450 are an essential tool in these studies. Several cytochromes P-450 have been substantially purified in a one-step immunoadsorption procedure using Sepharose bound MAbs to the major forms of rat liver cytochrome P-450 induced by 3-methylcholanthrene and phenobarbital (MC-P-450 and PB-P-450, respectively). When mixed with solubilized tissue microsomes, the immunoadsorbents bind polypeptides which are readily desorbed at pH 3.0. Cytochromes P-450 have been purified with MAbs 1-7-1 and 1-31-2 from livers of C57Bl/67 and DBA/2 mice, guinea pigs and hamsters, and from rat lung. Such immunoadsorption experiments based on different MAbs reveal epitope relatedness between cytochromes P-450 in different tissues, strains, and species. The cytochromes P-450 isolated by this procedure were analyzed structurally by peptide mapping and NH_2-terminal amino acid analysis. Varying degrees of homology of these cytochromes P-450 were found. The hepatic cytochromes P-450 isolated from MC- and PB-induced rats exhibit small yet detectable levels of enzyme activity. Higher levels of active cytochromes P-450 were prepared by a novel antigen-exchange method in which inactive denatured P-450 was exchanged for native cytochrome P-450 bound to the immunoadsorbents.

The cytochromes P-450 metabolize a wide variety of drugs, chemicals and carcinogens. Specific modulators of aryl hydrocarbon hydroxylase (AHH) are useful probes for the study of the multiplicity, diversity, and different catalytic properties of the cytochromes P-450. We have examined the effect of a number of flavones on the catalytic activity of rat hepatic microsomes. The compounds may be classified according to their activating or inhibitory effect on the constitutive and 3-methylcholanthrene (MC) induced AHH. One class of flavones, typified by 7,8-benzoflavone, activates constitutive AHH and inhibits the MC-induced AHH. A second class, typified by maackiain, exhibits effects which are the reverse of 7,8-benzoflavone. The flavones we screened may prove useful in studies which characterize the cytochrome P-450 content of different tissues, and should be useful in exploring the mechanism of action of these isozymes.

Immunochemical methods were used to isolate human cytochrome P-450 genes. Cytochrome P-450s were purified from rat liver and extensively characterized with regard to inducibility by xenobiotics and steroids, and catalytic activity. Rabbit anti-cytochrome P-450 antibodies were utilized to screen human cDNA expression libraries. Full length cDNAs were isolated and their sequences determined. These cDNAs were compared with their rodent P-450 homologs. The P-450 cDNA probes will be used to analyze the regulation of cytochrome P-450 in cultured human cells and lymphocytes. They will also

be utilized to examine human genetic differences. As a model system we will first attemt to characterize the gene coding for the human debrisoquine 4-hydroxylase. The polymorphism of this P-450 enzyme system in humans has been well documented.

Polycyclic aromatic hydrocarbons induce oxidative and conjugative enzymes in responsive animals and in human cells. The oxidative enzymes, i.e., cytochrome P-450s, generate active intermediates from procarcinogens and thus initiate chemical carcinogenesis. We propose to investigate this hypothesis by directly introducing molecularly cloned full length P-450 cDNAs into different cells and analyzing the extent of binding of the carcinogen to the cellular macromolecules and the incidence of tumors by challenging with appropriate carcinogens. The infectious eukaryotic expression vector, vaccinia virus, was previously shown to express enzymatically active foreign proteins that are transported to the appropriate subcellular site. We have introduced the mouse P1-450 and P3-450 genes into the recombination vector and are in the process of transferring these into the vaccinia virus to generate the infectious recombinant vaccinia virus.

Protein Section - Studies (1) the relationship between chromatin structure and gene expression, (2) mechanisms by which chromosomal proteins affect the structure and function of chromatin, and (3) the isolation and characterization of opal suppressor tRNA genes in higher eukaryotes and the regulatory role of the opal tRNA.

A method for the two dimensional display for restriction fragments of mammalian genomic DNA has been developed. The new procedure is more rapid, less cumbersome and far more reliable than previously described procedures. The approach was used in a study of the organization and distribution of repeated sequences in the mouse genome. The linkage relationships of these elements (the members of the Bam H1 superfamily of repeated sequences) were also determined. This approach has important applications in gene mapping and for the development of a new generation of restriction fragment polymorphism probes for genetic diseases.

In the nucleus of the cell the DNA is stored and packed in discrete nucleoprotein structures. Regulation of the information encoded in DNA is dependent on specific protein-nucleic interactions. The main aim of the Protein Section is to clarify the protein-nucleic acid interactions which affect the structure and regulate the function of chromatin, chromosomes and of specific genes. This broad goal is achieved by isolating specific chromosomal proteins, developing immunochemical assays for these proteins and immunocytochemical techniques to study the organization of specific chromosomal proteins in chromatin and chromosomes. An additional avenue of investigation involves in vitro studies on the binding of specific chromosomal proteins to various DNA structures. Genetic engineering approaches are a powerful and versatile approach to elucidate the cellular role of proteins. Therefore, genes for chromosomal proteins are isolated and characterized. The relation between the chromatin structure and gene expression of genes coding for cytochrome P-450 is investigated in detail.

In the past year we have produced antibodies against non-histone chromosomal proteins and developed immunoaffinity chromatographic techniques which allow the isolation of nucleoprotein complexes enriched in defined chromosomal proteins. We have elucidated the mechanisms by which certain non-histone

chromosomal proteins bind to DNA as well as isolated a cDNA clone coding for a non-histone chromosomal protein.

The chromatin structure of genes coding for P-450 enzymes was investigated. Changes in the chromatin structure of these genes upon gene activation was found by comparing the chromatin structure of the genes in nuclei purified from the livers of both normal and inducer-treated rats. Micrococcal nuclease digestion revealed that one of the genes, P-450M, is present in a non-nucleosomal conformation. Four DNase I hypersensitive sites have been mapped in this gene. The sites do not change upon enzyme induction and are not present in tissues, such as rat thymus, which do not express this gene. The P-450M gene seems to be associated with the nuclear matrix. Using a plasmid containing the entire cloned P-450M sequence, an S1 sensitive site has been mapped. The DNA region recognizable by S1 may also be recognized by regulatory chromosomal proteins.

An evolutionary study of the sequence of opal suppressor tRNA genes and their flanking sequences (i.e., in human, rabbit, chicken and Xenopus genomes) has been completed. Previously, a chicken and a human opal suppressor tRNA gene were isolated and sequenced and this past year the sequences of the corresponding genes, which had been isolated from rabbit and Xenopus genomes, were determined. The human and rabbit genes are strictly conserved as are those of chicken and Xenopus; and the mammalian genes differ from the others by a single pyrimidine transition at position 11. The gene, therefore, has been highly conserved which provides strong evidence that the gene products have an important cellular function. In addition, two gene products which are known to form phosphoseryl-tRNA arise from the single opal suppressor tRNA gene. The fact that these tRNAs are phosphorylated on the serine moiety and read the nonsense codon UGA suggest that they have a specialized and unique function which lies outside our present concepts of tRNA utilization. The tRNA gene products are present in minor levels intracellularly compared to other seryl-tRNA isoacceptors. Both the 5' flanking region, which has been highly conserved between rabbit and human genomes and has short stretches of homology in all four species, and the 5' internal control region, which has two extra nucleotides compared to the corresponding segment in all known tRNA genes, may have a regulatory role in reducing the level of transcription of the opal suppressor tRNA genes.

Nucleic Acids Section - Studies (1) The nature of human genetic predisposition to cancer, (2) interaction of chemical and physical carcinogens with nucleic acids and their actions on the functions of DNA, (3) the relationship between defects in repair of cellular DNA and human cancer and (4) chemically produced alteration of DNA and the repair of such alterations.

The study of human cells defective in repairing damaged DNA was extended, with the rationale that DNA-repair deficient cells are more susceptible to the adverse effects of carcinogens than their repair-proficient counterparts. A group of 19 human tumors and eight SV40-transformed strains deficient in a 22,000 M.W. protein responsible for repairing O^6-MeG (a modified DNA base made by certain methylating agents) was identified earlier in this project. Such strains are called Mer^-. Viral transformation often produces Mer^- strains. Mer^+ strains contain 60,000 repair protein molecules per cell. A group of five Mer^+ cell strains, somewhat sensitive to cell killing by MNNG, repairs approximately one-third as much O^6-MeG as fully repair-proficient cell strains.

Many Mer$^-$ human tumor lines were determined to be more sensitive to cell killing by human interferons than were Mer$^+$ human tumor cell lines, indicating a possible common origin of sensitivities to both agents. Experimental modulation by interferon treatment of oncogene transcription was shown to modulate the cellular ability to repair O^6-methylguanine in NIH3T3 cells, demonstrating for the first time a link between DNA repair and oncogene activation.

The retrospective study of xeroderma pigmentosum (XP), a genetically recessive human disorder predisposing to skin cancer of sun-exposed areas continues. The results indicated that XP patients are 2000-fold more susceptible to skin cancer than the general U.S. population and that the age at onset of skin neoplasia is 50 years earlier in XP patients than in the general population. Further, persons with XP have increased susceptibility to brain sarcomas. A new host-cell reactivation assay utilizing plasmid transfection techniques was used to investigate the UV-repair defect characteristic of XP. One photoproduct per plasmid-borne chloramphenicol acetyltransferase gene inactivates the gene when the plasmids enter XP cells.

A shuttle vector plasmid shows a different spectrum of UV-induced mutations when introduced into XP than in normal cells. This new approach utilizing shuttle vectors shows great promise for understanding the molecular basis for defective DNA repair in XP cells. The shuttle vector plasmid was constructed which carries a small marker gene and which replicates in human bacterial cells. Plasmids treated with ultra violet light were introduced into repair-proficient and repair-deficient human cells. Plasmids with UV induced mutation in the marker gene were identified and characterized in a sensitive micro biological assay. The precise nature of the mutations was determined by DNA sequence analysis. The nature and location of changes were different in the repair-proficient and -deficient cells. This is the first molecular determination of carcinogen induced mutations in human cells.

Actins are major components of all eukaryotic cells. Functionally they are involved in the cytoskeletal structures and these structures are altered in transformed cells. To understand the structure and expression of the human actin genes, we have begun a molecular analysis of molecularly cloned human actin genes. DNA sequence analysis of the human smooth muscle actin gene (aortic type) showed that this gene has a unique intron not found in any other actin genes analyzed, suggesting that this intron was inserted late in the evolutionary process. The smooth muscle actin gene in chemically transformed human cells showed a point mutation not found in the normal cell counterpart.

To study whether the cloned human cardiac actin DNA contained sequences necessary for its expression, a recombinant plasmid was constructed by inserting the cardiac actin DNA into the expression shuttle vector bovine papilloma viral DNA. Mouse cells transformed with this recombinant expressed a substantial amount of the human cardiac mRNA and this mRNA was faithfully translated into cardiac actin in mouse cells. The results indicate that the cloned actin DNA contains (within its 5' and 3' untranslated sequences) signals for the initiation and termination of transcription as well as for translation. Bovine papilloma viral DNA vectors can be used to stably express cloned human DNA sequences in mouse cells.

The high mutagenic frequencies in bacterial strains indicate that fecapentaene-12 (fec-12) is a direct mutagen and it may be enzymatically deactivated by the S9

fraction. The reannealing of heat denatured linear pBR322 DNA is significantly more rapid with fec-12 treated DNA than untreated DNA, a phenomenon similar to that due to nitrous acid and trimethylpsoralen plus UV irradiation. Single and double stranded DNA treated with fec-12 demonstrates a high intensity of fluorescence, suggesting covalent binding. Alkaline elutions of fec-12 treated fibroblast DNA also show dose and time dependent single strand breaks and cross-linking of the DNA, supporting the findings from *in vitro* studies. These results indicate that fec-12 causes DNA damage in human cells which results in cytotoxicity and mutation.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP04496-08 LMC

PERIOD COVERED

October, 1984 to September 30, 1985

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Chromosomal Proteins and Chromatin Function

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	Michael Bustin	Acting Section Chief	LMC	NCI
Others:	David Landsman	Visiting Fellow	LMC	NCI
	Shulamith Druckmann	Visiting Fellow	LMC	NCI
	Leo Einck	Senior Staff Fellow	LMC	NCI

COOPERATING UNITS (if any)

Department of Biochemistry, Georgetown University, (Dr. M. Smulson)
Department of Zoology, University of Massachusetts, Amherst, (Dr. C. Woodcock)

LAB/BRANCH

Laboratory of Molecular Carcinogenesis

SECTION

Protein Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.6	2.1	0.5

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The role of chromosomal proteins in maintaining the structure and regulating the function of chromatin and chromosomes is studied. Antibodies against a specific fraction of chromosomal nonhistone proteins have been elicited and the major antigenic entities identified. The binding of monoclonal antibodies to histone H5 and H1° has been mapped in detail. Chromatin fragments highly enriched in HMG-17 and H1° have been purified by immunoaffinity chromatography. The exchange of chromosomal proteins among various chromatin fragments has been studied. Details of the interactions of chromosomal proteins HMG-1 and HMG-2 with various DNA structures have been elucidated. The results indicate that chromosomal proteins HMG-1 and HMG-2 are single-stranded DNA binding proteins which can distinguish between similar DNA structures. These proteins constitute a component that is obligatory for proper cell function. They serve as structural elements maintaining specific chromatin regions in a particular conformation which may be recognizable by regulatory elements.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged in this Study:

Michael Bustin	Acting Section Chief	LMC	NCI
David Landsman	Visiting Fellow	LMC	NCI
Shulamit Druckmann	Visiting Fellow	LMC	NCI
Leo Einck	Senior Staff Fellow	LMC	NCI

Objectives:

To understand the role of defined chromosomal proteins in maintaining the structure and regulating the function of chromatin and chromosomes in normal and transformed cells.

Methods Employed:

In the nucleus of the cell the DNA is stored and packed in discrete nucleoprotein structures. Regulation of the information encoded in the DNA is dependent on specific protein-nucleic acid interactions. The chromatin fiber and the protein nucleic acid interactions in the fiber are in a dynamic state. To facilitate the study of specific chromosomal proteins, their interactions with DNA and their cellular function, we have elicited antibodies against specific proteins and used the antibodies to study the structure and function of chromatin. Proteins were purified from isolated nuclei by ion exchange chromatography, size exclusion chromatography and differential precipitation. Polyclonal antibodies are elicited in rabbits. Monoclonal antibodies were elicited in mice. Chromatin was isolated from purified nuclei. The antigenic activity of the isolated proteins and of the purified chromatin was measured by ELISA radioimmune assay and immunoblotting. The Ig fraction was purified by chromatography on affy-blue columns. Affinity columns were prepared by the cyanogen bromide procedure. Plasmids were propagated in HB101 and purified by two centrifugations through $CsCl_2$. The purified DNA was radioactively labelled by nick-translation or by primer extension.

In vivo labelling of plasmids is achieved by growing the plasmids in the presence of ^{32}P orthophosphate. The binding of proteins to DNA is measured by filter binding. Cloning of the HMG gene is done in λgt11 and the recombinant phages which express the HMG protein are identified with polyclonal antibodies.

Major Findings:

1. Immunological Probes for Chromatin

   Antibodies to a class of nonhistone proteins have been elicited in mice. These antibodies specifically recognize a class of proteins which is present in several transformed human cell lines. Analysis of this fraction by electrophoresis on polyacrylamide gels reveals that the protein components differ among the cell lines; however, this difference is not reflected in the antigenic properties of the fraction since the fractions obtained from different cell lines gave identical immunoblotting patterns. These antisera

will be useful in studying the cellular role of these nonhistone proteins. A battery of 11 monoclonal antibodies against histone H_5 and H1° has been elicited and the epitopes to which these antibodies bind have been identified. Two of these monoclonal antibodies are used to study the organization of histone H_5 in chromatin by immunoelectron microscopy and by immunosedimentation. Monoclonal 3H9 binds very strongly to chromatin while the binding of monoclonal IC3 is sterically hindered. The binding is highly influenced by ionic strength. At low ionic strength, the binding is more efficient than at higher ionic strength suggesting that the H_5 is placed on the inside the solenoidal structure of chromatin. These studies will help in elucidating the detailed organization of the higher order chromatin fiber.

2. Fractionation of Chromatin by Immunoaffinity Chromatography

Purification of chromatin fragments containing specific chromosomal proteins will ultimately reveal whether specific chromosomal proteins such as H1° and HMG-17 are associated with specific DNA sequences. The validity of such studies required proof that the proteins did not migrate and rearrange during the various manipulations.

The migration and rearrangement of chromosomal proteins during immunofractionation of chromatin has been investigated. Oligonucleosomes from two different chromatins, chicken erythrocyte or rat liver, were mixed with oligonucleosomes from the other species which had been depleted of histones H1/H5 and high mobility group proteins (HMGs). The mixture was treated with buffers of various ionic strengths and immunofractionated on an anti-HMG-17 IgG-Sepharose column. The type of DNA, which was retained as the bound fraction on the column, was determined by slot blot analysis using nick-translated repetitive DNA probes from either chicken or rat. The results indicate that in low ionic strength buffers (i.e., below 40 mM NaCl), there is very little exchange of either histone H5 or HMG-17 among nucleosomes and therefore, we suggest that it is possible to fractionate nucleosomes according to their antigenic content.

Recently, we have isolated polynucleosomes enriched in HMG-17 and H1°. The DNA from the polynucleosomes is purified and examined for the abundance of repetitive sequences, transcribed sequences, nontranscribed sequences and sequences coding for genes which are inducible by treatment with chemical carcinogens.

3. Binding of Chromosomal Proteins HMG-1 and HMG-2 to Superbilical DNA

Chromosomal proteins HMG-1 and HMG-2 are proteins which preferentially bind single-stranded DNA. They are ubiquitously distributed in all tissues in all the eukaryotic kingdoms and most probably are involved in determining structural characteristics of chromatin.

The interaction of chromosomal proteins HMG-1 and HMG-2 with various DNA structures has been examined with plasmid pPst-0.9, which contains DNA sequences that can form the Z-DNA conformation and palindromic sequences

that can form cruciform structures. Direct binding and competition experiments with a ^{32}P-labeled plasmid indicated that proteins HMG-1 and HMG-2 preferentially bind to supercoiled form I DNA as compared to double-stranded linear DNA. The preferential binding to form I is due to the presence of single-stranded regions in this DNA. The binding of HMG-1 and HMG-2 to the form I plasmid results in inhibition of S_1 nuclease digestion in a selective manner. The B-Z junction is preferentially protected as compared to the cruciform, which in turn is more protected than other minor S_1-sensitive structures present in pPst-0.9. Our results indicate that the binding of HMG-1 and HMG-2 proteins to DNA is not random in that HMG-1 and HMG-2 can distinguish between various S_1 nuclease sensitive sites in the plasmid. The existence of a hierarchy of DNA binding sites for these proteins suggests that they can selectively affect the structure of distinct regions in the genome.

Recently, we found that HMG-2 binds to DNA in a different manner than HMG-1. The results suggest that these two closely related proteins perform distinguishable cellular functions.

4. Cloning the HMG-17 Gene

Chromosomal protein HMG-17 maintains transcribable DNA sequences in a conformation which is different from that of bulk chromatin. To gain further information on the cellular role and mode of action of this protein, it is necessary to clone the gene and study its structure, regulation and arrangement in normal and transformed cells. We have taken advantage of the anti-HMG-17 antibodies which we have elicited and used to screen a λgt11 human c-DNA library. Several clone codings for HMG-17 have been detected. Isolation of the DNA from these clones and sequencing of the DNA is in progress.

Significance to Biomedical Research and the Program of the Institute:

Understanding the mechanism of gene regulation and its relation to neoplasia requires knowledge of the structure of chromatin and chromosomes and information on the manner in which the genetic information encoded in DNA is expressed in a controlled fashion. Specific interactions between chromosomal proteins and the nucleic acid component of the genome are key mechanisms in determining the structure and function of chromatin. Presently, the approach developed in this laboratory is the only approach in which specific probes for well-defined, purified chromosomal components are used to study the organization of these components in intact chromatin and chromosomes. As such, a unique opportunity has developed whereby certain structural aspects of these nucleoproteins can be visualized and directly related to functional stages of the genome. Furthermore, the specific antibodies serve as reagents to facilitate cloning of the genes coding for chromosomal proteins. Isolation of the genes coding for these proteins will allow the ease of genetic engineering techniques for the study of the cellular role of these ubiquitous chromosomal proteins.

Proposed Course:

Studies aimed at elucidating the structure-function relation of chromosomal proteins will continue. In the forthcoming year we will concentrate the efforts on:

1. Characterizing the DNA associated with chromosomal proteins HMG-17 and H1°.

2. Elucidating the manner in which H1° is bound to the core chromatin particles.

3. Cloning the HMG-17 gene.

Publications:

Bustin, M.: Chromosomal nonhistone proteins. Am. Sci. 72: 631-632, 1984.

Bustin, M.: Immunological studies on the structure and function of HMG proteins. In Bekhor, I. (Ed.): Progress in Nonhistone Chromosomal Protein Research. CRC Press, 1985, Vol. 11, pp. 75-90.

Chapelear, M. S., Bustin, M., and Glazer, R. I.: Evidence that HMG17 is not phosphorylated in human colon carcinoma cells. Biochim. Biophys. Acta. 838: 351-354, 1985.

Dunn, B., Mendelson, E., Soares, N., and Bustin, M.: Antigenicity of 5M urea soluble chromosomal proteins from Hela cells. Biochim. Biophys. Acta. 838: 151-160, 1985.

Einck, L. and Bustin M.: The intracellular distribution and function of HMG proteins. Exp. Cell Res. 156: 295-310, 1985.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP04516-09 LMC

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Cellular and Molecular Effects of Psoralen Plus Ultraviolet Light

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI: K. H. Kraemer Senior Surgeon LMC NCI

COOPERATING UNITS *(if any)*

None

LAB/BRANCH

Laboratory of Molecular Carcinogenesis

SECTION

Cell Genetics Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.1	0.1	0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Psoralen plus long wavelength ultraviolet radiation (UV-A) is being investigated as a model system for clinically relevant photochemical carcinogenesis and as a probe for defective DNA repair. Used experimentally for treatment of psoriasis and mycosis fungoides, psoralen plus UV-A has been found to be mutagenic, carcinogenic and immunosuppressive. We developed an in vitro assay to measure the effects of UV-A mediated psoralen-DNA binding in human lymphoid cells. Parameters monitored include the rate of DNA synthesis, induction of DNA-psoralen cross-links, induction of sister chromatid exchanges, alterations in the rate of cell proliferation and survival, and in immune reactivity. These studies indicate that the low doses of psoralen plus UV-A received by patients' leukocytes during therapy may result directly in decreased DNA synthesis in their circulating lymphoid cells. Cell survival was found to be markedly dependent on UV-A exposure and 8-MOP concentration, to be correlated with inhibition of DNA synthesis, and to be related to induction of DNA interstrand cross-links. There was a dose-dependent reduction in mixed leukocyte culture reactivity induced by 8-MOP plus UV-A treatment. Pathways for repair of psoralen DNA damage were examined by studying cells from a patient with the genetic disease, Cockayne's syndrome. Cells from a patient with Cockayne's syndrome had normal survival following 8-MOP plus UV-A treatment, but reduced survival after treatment with UV-B. Thus repair of photosensitized 8-MOP damage involves at least one pathway that is different from that for UV-B damage.

PHS 6040 (Rev. 1/84)

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

K. H. Kraemer Senior Surgeon LMC NCI

Objectives:

Humans are exposed to chemicals which may interact with ultraviolet radiation to become carcinogenic. A compound which is found in many plants, 8-methoxy-psoralen (8-MOP), plus high intensity long wavelength ultraviolet radiation (UV-A) is being used experimentally to induce remissions in psoriasis and in mycosis fungoides. The combination of 8-MOP plus UV-A produces DNA-8-MOP binding and has been shown to induce mutations in bacteria and in mammalian cells, to cause skin cancer in mice and in humans, and to be immunosuppressive. Individuals with some cancer-prone genetic diseases may be at increased risk from this treatment. We are developing an in vitro model system to assess clinically relevant photochemical carcinogenesis and to explore photoactivated psoralens as a probe for DNA damage and repair in human genetic diseases.

Methods Employed and Major Findings:

We previously demonstrated that circulating lymphoid cells of some psoriasis patients receiving 8-MOP plus UV-A therapy had a significant reduction in DNA synthesis. We have developed an in vitro assay system to approximate some of the conditions of 8-MOP plus UV-A exposure of human lymphoid cells during in vivo therapy. The assay has been used with fresh lymphocytes and with long-term lymphoblastoid cell lines. These results indicate that the low doses of 8-MOP and UV-A received by patients' lymphocytes during therapy may be sufficient to explain the decreased DNA synthesis found in their circulating lymphoid cells.

In the lymphoblastoid cells, as much as a 50% inhibition of DNA synthesis following 8-MOP plus UV-A treatment was associated with 100% survival. Greater inhibition of DNA synthesis resulted in an exponential decrease in cell survival. Similarly, measurements of 8-MOP-DNA cross-linking by the alkaline elution technique revealed a dose dependent increase in cross-link induction above a threshold of approximately 50% inhibition of DNA synthesis. The formation of detectable cross-links was also correlated with decreased cell survival. Thus, DNA-8-MOP interstrand cross-links may be responsible for inhibition of DNA synthesis and cell killing.

In vitro treatment of lymphocytes or lymphoblastoid cells with 8-MOP plus UV-A resulted in approximately a doubling in the number of sister chromatid exchanges per metaphase. Further increases in dosage of 8-MOP plus UV-A were toxic. Thus, it is likely that the doses of 8-MOP plus UV-A received by patients' lymphocytes are too low to permit routine detection of increased sister chromatid exchanges. Mixed leukocyte reactivity of fresh human leukocytes was found to be inhibited in a dose dependent manner by 8-MOP plus UV-A in vitro. Stimulator and responder functions were both inhibited. This inhibition of immune reactivity may be exploited to provide immunosuppression.

Cultured cells from patients with Cockayne's syndrome (CS) are hypersensitive to the growth-inhibiting effects of sunlight (UV-B radiation). Cells from one CS patient are hypersensitive to UV-B but have a normal proliferative response to photosensitized 8-MOP. This implies that there is at least one human cellular recovery pathway that is different for UV-B and for photosensitized 8-MOP.

Significance to Biomedical Research and the Program of the Institute:

These results indicate that the low doses of 8-MOP and UV-A received by patients' lymphocytes during therapy may be sufficient to explain the decreased DNA synthesis found in their circulating lymphoid cells. The fact that Cockayne's syndrome cells are hypersensitive to UV-B but not to 8-MOP plus UV-A indicates that photosensitized psoralen damage may, in part, be handled by human cellular recovery pathways different from that for sunlight-induced damage.

Proposed Course:

This project is terminated.

Publications:

Kraemer, K. H. and Waters, H. L.: Effects of psoralens plus ultraviolet radiation on human lymphoid cells *in vitro*. *Natl. Cancer Inst Monogr*. 66: 221-223, 1984.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP04517-09 LMC

PERIOD COVERED
October 1, 1984 to September 30, 1985

TITLE OF PROJECT (*80 characters or less. Title must fit on one line between the borders.*)
DNA Repair In Human Cancer-Prone Genetic Diseases

PRINCIPAL INVESTIGATOR (*List other professional personnel below the Principal Investigator.*) (*Name, title, laboratory, and institute affiliation*)

PI: K. H. Kraemer, Sen. Surgeon, LMC NCI

Others:
- M. Protic-Sabljic, V Fellow, LMC NCI
- A. Bredberg, V Fellow, LMC NCI
- M. H. Greene, Med. Officer, EEB NCI
- J. Scotto, Biometrician, BB NCI
- B. Howard, Section Head, LMB NCI
- R. Day, III, Sec. Head, LMC NCI
- G. Peck, Sr. Invest., DB NCI
- M. Seidman, Expert, LMC NCI
- J. Robbins, Sr. Invest., DB NCI

COOPERATING UNITS (*if any*) Dept. of Path., NJ Med. Sch., Newark, NJ (W. C. Lambert); Dept. of Derm., Columbia U., NY, NY (A. D. Andrews); NY Blood Ctr., NY, NY; (J. L. German); Dept. Derm., Hosp. U of Penn., Phila. PA (W. H. Clark); Tel Aviv U., Tel Aviv, Israel (H. Slor)

LAB/BRANCH
Laboratory of Molecular Carcinogenesis

SECTION
Cell Genetics Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
4.5	3.5	1.0

CHECK APPROPRIATE BOX(ES)

- [x] (a) Human subjects
  - [x] (a1) Minors
  - [ ] (a2) Interviews
- [x] (b) Human tissues
- [ ] (c) Neither

SUMMARY OF WORK (*Use standard unreduced type. Do not exceed the space provided.*)

Patients with xeroderma pigmentosum (XP) and ataxia-telangiectasia (A-T), diseases with ultraviolet (UV) and x-ray hypersensitivity, respectively, and with the dysplastic nevus syndrome of hereditary cutaneous melanoma (DNS) are being studied. Detailed examinations of the clinical features of affected individuals are being made. A prospective registry of XP patients is under way. Field studies in Israel detected a possible new form of XP with defective DNA repair without neoplasia. Patients with XP and a high frequency of skin cancer are being treated with 13 cis-retinoic acid in an attempt to prevent formation of new skin cancers. We demonstrated the suitability of SV40 virus-transformed XP cells as high efficiency recipients of transfected plasmids. XP cells stably integrated many copies of transfected genes and expressed linked genes with high frequency. We developed a host cell reactivation DNA repair assay that measures transient expression of chloramphenicol acetyl transferase (CAT) activity in XP and normal cells transfected with a UV-treated plasmid bearing the CAT gene. In the XP cells we showed that one pyrimidine dimer blocks expression of the transfected CAT gene. Studies in progress are measuring the sites and types of mutations induced by ultraviolet radiation in shuttle vector plasmids replicated in XP and normal cells. Collaborative clinical studies of 14 DNS kindreds have demonstrated autosomal dominant inheritance of the melanoma trait, new melanomas only in family members with dysplastic nevi and >100-fold increased melanoma risk in family members with dysplastic nevi. Similar dysplastic nevi also occur in non-familial settings. A new classification of DNS was proposed. Laboratory studies demonstrated UV-induced hypermutability in DNS lymphoblastoid cell lines and normal DNA repair. A-T is an autosomal recessive disorder with clinical and cellular x-ray hypersensitivity and a high incidence of neoplasms. Lymphoblastoid cell lines established from 10 kindreds with A-T are being tested for heterozygote detection.

PHS 6040 (Rev. 1/84)

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

K. Kraemer	Senior Surgeon	LMC NCI
M. Protic-Sabljic	Visiting Fellow	LMC NCI
A. Bredberg	Visiting Fellow	LMC NCI
M. H. Greene	Medical Officer	EEB NCI
J. Scotto	Biometrician	BB NCI
B. Howard	Section Head	LMB NCI
M. Seidman	Expert	LMC NCI
R. Day, III	Section Head	LMC NCI
G. Peck	Senior Investigator	DB NCI
J. Robbins	Senior Investigator	DB NCI

Objectives:

Human cancer-prone genetic diseases are being studied with a view toward identifying groups of people with an increased susceptibility to environmental carcinogenesis. We are attempting (1) to correlate such sensitivity with clinical abnormalities, (2) to determine if there is genetic diversity within such groups, (3) to understand the molecular basis of their cellular hypersensitivity, (4) to develop tests to identify persons at increased risk of neoplasia, (5) to explore methods of cancer prevention in these patients, and (6) to educate the medical community as to the importance of early recognition and diagnosis of these disorders.

Methods Employed:

Patients are examined with particular emphasis on cutaneous abnormalities, and cultures of skin fibroblasts or peripheral blood lymphocytes are established for laboratory analysis. Patients with xeroderma pigmentosum (XP), dysplastic nevus syndrome of familial cutaneous malignant melanoma (DNS) and ataxia-telangiectasia (A-T) have been studied clinically.

The English language medical literature on XP is being reviewed comprehensively, and information on individual patients abstracted and entered into a computer for analysis. Physicians treating patients with XP have been contacted and are encouraged to fill out a Xeroderma Pigmentosum Registry questionnaire about their patients. New clinical forms of XP are investigated in depth. XP patients with multiple cutaneous neoplasms are being treated with 13-cis retinoic acid to attempt to reduce the rate of tumor formation.

DNA mediated gene transfer (transfection) is being used to introduce genes into XP cells in expression vector plasmids. Stable integration and transient expression is measured. Inactivation of expression vector plasmids form the basis for a new host cell reactivation assay of DNA repair. Shuttle vector plasmids are being utilized to study the sites and base changes induced by ultraviolet radiation in plasmids replicated in XP and normal cells. Melanocytes and nevus cell from pigmented lesions in XP are cultured and studied for DNA repair.

Clinical diagnostic features of DNS are being refined. Lymphoblastoid cell lines from patients with familial DNS are being studied for ultraviolet induced mutagenesis, and the effect of UV on cell survival, DNA synthesis and DNA repair.

Major Findings:

XP is an autosomal recessive cancer-prone disease with clinical UV hypersensitivity, accompanied by cutaneous and neurological abnormalities. Cultured cells from XP patients have cellular UV sensitivity and defective DNA repair. We have compiled the most comprehensive review of the world literature to date on XP including both clinical and laboratory observations. Data on more than 800 XP patients described in the literature have been entered into the computer. In this retrospective study, we have documented reduction of 50 years in the age of onset of skin neoplasms in XP in comparison to the U.S. population. There is a 2000-fold increase in all three major types of skin neoplasms: basal cell carcinomas, squamous cell carcinomas, and melanomas. The XP melanoma site distribution does not correspond to areas of greatest sun exposure implying that factors other than UV may be important in melanoma. Oral cavity neoplasms are increased, possibly due to UV exposure of the anterior tongue and/or to dietary carcinogens. We found lymphoblastoid cell lines from XP patients to be hypersensitive to killing by tryptophane pyrolysis products (carcinogens produced in charbroiled foods). Internal neoplasms, particularly sarcoma of brain, were found at increased frequency. A registry of XP patients is being established. In a collaborative study with H. Slor of the Tel Aviv University in Israel, we identified kindreds with a form of XP that has not been previously recognized. Clinically, facial lesions predominate. One family member has reduced DNA repair with minimal clinical symptoms, while a brother with equally reduced DNA repair has numerous neoplasms. This suggests that other, presently unmeasured, factors may be crucial to the development of neoplasms in XP.

In our attempts to clone the genes responsible for UV hypersensitivity in XP cells, we have collaborated with the Laboratory of Molecular Biology (see Project Number Z01CB08719-05 LMB) and developed a protocol that gives high efficiency (about 10^{-3}) transfection of cloned selectable genes into XP cells. We found that SV40-transformed XP cells, but not primary fibroblasts, are suitable recipients of cloned genes. XP cells were transfected with a plasmid containing two genes: chloramphenicol acetyl transferase (CAT) and xanthine phosphoribosyl transferase (XPRT). Eighty to 90% of cell clones selected for ability to express XPRT also expressed CAT. The XP cells integrated greater numbers of copies of transfected genes than did the normal cells.

A host cell reactivation assay demonstrated reduced expression of UV damaged expression vector plasmids in XP cells. XP group A cells had more than 100-fold less CAT activity than control cells with some doses of UV. There were similar UV-inactivation curves for 3 plasmids of different sizes and promoters containing the same gene indicating that the target was the gene. In the XP cells one pyrimidine dimer per gene inactivates expression of the transfected gene.

Shuttle vector plasmids demonstrate that the spectrum of mutations found in UV treated plasmids replicated in XP cells is different from that in the normal cells. There is a marked reduction in the locations of the mutations and a narrower spectrum of types of base changes found in the XP group A cells.

A newly recognized clinical disease, familial malignant melanoma with a characteristic precursor lesion, the dysplastic nevus, is being examined in a collaboration with the Environmental Epidemiology Branch, NCI (Z01CP04410-09 EEB). This laboratory is contributing dermatological expertise to the clinical definition of the syndrome in a study of more than 400 family members in 14 kindreds with an average 6 year follow-up. DNS family members with these distinctive nevi were found to have several hundred-fold increased risk of developing cutaneous melanoma. Other findings include autosomal dominant inheritance of the melanoma trait, new melanomas only in family members with dysplastic nevi, a young age at diagnosis of melanoma, and a high frequency of multiple primary melanomas. We estimated that, in the U.S., about 32,000 people have familial DNS representing about 6% of the melanomas. Similar dysplastic nevi also occur in non-familial settings involving an estimated several million people in the U.S. A new classification of DNS was proposed, emphasizing that the most numerous group (sporadic DNS without melanoma) is probably at lowest melanoma risk.

Lymphoblastoid cell lines from selected DNS patients are being examined for evidence of sensitivity to DNA agents as measured by cell survival and mutagenesis and examined for possible DNA repair defects. We found lymphoblastoid cell lines from DNS patients to be hypermutable by UV. Cell survival, rate of DNA synthesis, and rate of formation of DNA strand breaks following UV (measured by alkaline elution) were normal. The finding of hypermutability of non-cutaneous cells in DNS indicates that DNS is a generalized disorder. Further, DNS is only the second hypermutable human disease to be recognized.

A-T, an autosomal recessive cancer-prone disease with cutaneous, neurological, and immunological abnormalities, has x-ray sensitivity. We are studying the ability of cultured cells from A-T patients and their parents to survive DNA damage induced by the chemotherapeutic agent, bleomycin. This agent also induced an abnormally large increase in chromosome breakage (but not in sister chromatid exchanges) in A-T homozygous lymphoblastoid cells but not in heterozygous cells. DNA synthesis in A-T homozygotes, but not heterozygotes, was found to be resistant to treatment by x-ray or bleomycin in comparison to the response of normal cells. A collaborative study is in progress to detect A-T heterozygotes in coded lymphoblastoid cell lines from 5 kindreds by use of x-ray survival measurements.

Significance to Biomedical Research and the Program of the Institute:

These studies may identify persons with increased risk of cancer, may be useful in revealing the mechanism of cancer induction, and may suggest modes of cancer prophylaxis. In addition, these diseases serve as models for studies of human environmental carcinogenesis.

Proposed Course:

This project will be continued along the lines indicated above.

Publications:

Greene, M. H., Clark, W. H., Jr., Tucker, M. A., Elder, D. E., Kraemer, K. H., Guerry, D. IV, Witmer, W. K., Thompson, J., Matozzo, I, and Fraser, M. C.: Acquired precursors of cutaneous malignant melanoma: The familial dysplastic nevus syndrome. N. Engl. J. Med. 312: 91-97, 1985.

Greene, M. H., Clark, W. H., Jr., Tucker, M. A., Kraemer, K. H., Elder, D. E., and Fraser, M. C.: The prospective diagnosis of malignant melanoma in a population at high risk: Hereditary melanoma and the dysplastic nevus syndrome. Ann. Int. Med. 102: 458-465, 1985.

Kraemer, K. H.: Cellular hypersensitivity and DNA repair. In Fitzpatrick, T. B., Eisen, A. Z., Wolff, K., Freedberg, M. M. and Austen, K. F. (Eds.): Dermatology in General Medicine. New York, McGraw-Hill. (In Press)

Kraemer, K.H.: Xeroderma pigmentosum. In Provost, T.T. and Farmer, E.R. (Eds.): Current Therapy in Dermatology. Philadelphia, B.C. Decker, Inc., 1985, pp 172-174.

Kraemer, K. H. and Greene, M. H.: Dysplastic nevus syndrome: Familial and sporadic precursors of cutaneous melanoma. Dermatol. Clin. 3: 225-237, 1985.

Kraemer, K. H., and Slor, H.: Xeroderma pigmentosum. Clin. Dermatol. 3: 33-69, 1985.

Lambert, W. C., Andrews, A. D., German, J., and Kraemer, K. H.: Etiology and pathogenesis of xeroderma pigmentosum. In Dobson, R.L. and Thiers, B.H. (Eds.): The Etiology of Skin Diseases. New York, Churchill-Livingston Co. (In Press)

Protic-Sabljic, M., Whyte, D. B., Fagan, J., Howard, B. H., Gorman, C. M., Padmanabhan, R., and Kraemer, K. H.: Quantification of expression of linked cloned genes in an SV40 transformed xeroderma pigmentosum cell line. Mol. Cell. Biol. (In Press)

Protic-Sabljic, M., Whyte, D. B., and Kraemer, K. H.: Hypersensitivity of xeroderma pigmentosum cells to dietary carcinogens. Mutat. Res. 145: 89-94, 1985.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP04525-13 LMC

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Studies on Electrophoretic Techniques for Protein, RNA, and DNA

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Andrew C. Peacock	Chief, Protein Section	LMC	NCI
Others:	Sylvia L. Bunting	Research Chemist	LMC	NCI
	Michael Seidman	Senior Staff Fellow	LMC	NCI

COOPERATING UNITS *(if any)*

None

LAB/BRANCH

Laboratory of Molecular Carcinogenesis

SECTION

Protein Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.1	1.1	1.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

A method for the two dimensional display for restriction fragments of mammalian genomic DNA has been developed. The new procedure is more rapid, less cumbersome and far more reliable than previously described procedures. The approach was used in a study of the organization and distribution of repeated sequences in the mouse genome. The linkage relationships of these elements (the members of the Bam H1 superfamily of repeated sequences) were also determined. This approach has important applications in gene mapping and for the development of a new generation of restriction fragment polymorphism probes for genetic diseases.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

Andrew C. Peacock	Chief, Protein Section	LMC	NCI
Michael Seidman	Senior Staff Fellow	LMC	NCI
Sylvia L. Bunting	Research Chemist	LMC	NCI

Objectives:

Current efforts are directed towards finding ways to study the rearrangement of sequences in the human genome which may occur during carcinogenesis. The two dimensional analysis technique permits a substantially greater degree of resolution than previously possible and expands the collection of hybridization probes available for these studies.

Methods Employed:

Tissue culture, hybridization, gel electrophoresis, restriction analysis of DNA.

Major Findings:

The mouse Bam H1 superfamily of repeated sequences was found to be more heterogeneous than previously described. A major component of this family is the Eco R1 1.3 kb element which was shown to exist in a variety of environments defined by restriction digestion. These include the Bam H1 4 kb fragment but also a non continuous range of Bam H1 fragments from 1.0 kb to ca 30 kb. In addition, several other fragment series were identified in which sequences homologous to the 1.34 kb element were present but of variable length. The study also demonstrated the linkage relationships between various components of the Bam H1 4 kb family. The results suggest that some components are much more disperse than others, suggesting that the more disperse elements are older than those with a more restricted distribution. This approach was also applied to several other sequences of variable copy number in mammalian genomes.

Significance to Biomedical Research and the Program of the Institute:

Although two-dimensional analyses of DNA would appear to be a powerful tool with many applications, it is seldom used. This is because previously published procedures for generating continuous two-dimensional patterns have been tedious and unreliable. This is the first reliable method for continuous display of genomic fragments. The detail and resolution of genomic components permitted by this procedure have never been described before. The technique will be applied to problems in genomic mapping and identification and characterization of genomic rearrangements. Furthermore, it should permit the expansion of the repertoire of probes for defining restriction fragment polymorphisms that are diagnostic of genetic diseases.

Proposed Course:

To examine the utility of this system with a varieties of genomic materials from bacterial and mammalian cells.

Publications:

Peacock, A.C., Bunting, S.L., Cole, S.P.C. and Seidman, M.I.: Two-dimensional electrophoretic display of restriction fragments from genomic DNA. Anal. Biochem. (In Press)

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP04578-09 LMC

PERIOD COVERED

October 1, 1984 through September 30, 1985

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Structure and Expression of Human Actin Genes

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	N. Battula	Expert	LMC	NCI
Others:	J. Sagara	Visiting Fellow	LMC	NCI
	T. Kakunaga	Visiting Scientist	LMC	NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Molecular Carcinogenesis

SECTION

Cell Genetics Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.5	1.5	0.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Actins are major components of all eukaryotic cells. Functionally they are involved in the cytoskeletal structures and these structures are altered in transformed cells. To understand the structure and expression of the human actin genes, we have begun a molecular analysis of molecularly cloned human actin genes. DNA sequence analysis of the human smooth muscle actin gene (aortic type) showed that this gene has a unique intron not found in any other actin genes analyzed, suggesting that this intron was inserted late in the evolutionary process. The smooth muscle actin gene in chemically transformed human cells showed a point mutation not found in the normal cell counterpart.

To study whether the cloned human cardiac actin DNA contained sequences necessary for its expression, a recombinant plasmid was constructed by inserting the cardiac actin DNA into the expression shuttle vector, bovine papilloma viral DNA. Mouse cells transformed with this recombinant expressed a substantial amount of the human cardiac mRNA and this mRNA was faithfully translated into cardiac actin in mouse cells. The results indicate that the cloned actin DNA contains (within its 5' and 3' untranslated sequences) signals for the initiation and termination of transcription as well as for translation. Bovine papilloma viral DNA vectors can be used to stably express cloned human DNA sequences in mouse cells.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged in this Project:

N. Battula	Expert	LMC	NCI
J. Sagara	Visiting Fellow	LMC	NCI
T. Kakunaga	Visiting Scientist	LMC	NCI

Objectives:

The overall goal is to understand the role of actins in the cytoskeletal structures of normal and transformed cells. Specific aims are to 1) characterize the structural organization of the actin DNA sequences and 2) define the regulatory sequences of the actin DNA responsible for transcription, translation and tissue specific expression.

Methods Employed:

The project involves the isolation of actin DNA sequences from human cell genome, characterization of the isolated actin DNAs and introduction of these genes into cells to study the control of their expression. To achieve this, a variety of biological and biochemical techniques were employed. For example: 1) Human Hut 14 cell genomic DNA library made in phage © was screened by nucleic acid hybridization using nick translated actin specific labeled probes and the actin specific sequences isolated. 2) The full length and subgenomic fragments of the actin DNAs were subcloned into different vectors by employing recombinant DNA techniques for analysis and preparation of large amounts of pure DNAs. 3) To determine the primary structure of the actin DNA, nucleotide sequencing was carried out. 4) To check if the cloned actin DNA sequences have all the information required for their expression, the actin DNAs were transposed onto the expression vector, bovine papilloma viral DNA and the recombinant was introduced into the mouse cells by DNA transfection and the transformants possibly producing actin were selected by the transformed phenotype. 5) To determine the status of the introduced genes in the mouse cell transformants and characterize their specific expression products, the cellular nucleic acids were electrophoresed, the DNAs and mRNAs analyzed by Southern and Northern blots. The protein products were identified by 2-dimensional gel electrophoresis.

Major Findings:

A restriction map of the genomic DNA clone of the human smooth muscle actin gene (Aortic type) was generated. The primary nucleotide sequencing of the human smooth muscle actin gene and its intron and exon positions was determined. This gene was found to contain an additional unique intron site not found in other actins thus far studied. This unique intron's location lead to a hypothesis that this insertion or transposition occurred at relatively recent times in evolution. There was a single transition point mutation in the smooth muscle actin of this chemically transformed human cell line in agreement with the reported occurrences of point mutations in chemically transformed cells.

By sequence analysis, human cardiac muscle actin DNA was previously shown to contain 5 introns interrupting the coding exons. However, it is not known whether or not the 5' and 3' untranslated sequences of this fragment harbor the regulatory sequences required for the expression of the coding sequences. To test this, the actin DNA was inserted into the expression vector, bovine papilloma viral DNA, and introduced into the mouse cells. The results showed that a) the recombinant DNA was stably retained in an episomal state as a free replicating plasmid with 10-50 copies per cell, b) the transformed cells transcribed large amounts of cardiac actin mRNA of the expected size and the transcription occurred regardless of the orientation of the actin DNA, c) the transformed cells accumulated substantial amounts of the cardiac actin protein, and d) the human cardiac actin protein made in mouse cell transformants was incorporated into the cytoskeletal structures of the mouse cells. These results show that the molecularly cloned human cardiac actin DNA contains regulatory signals for the initiation and termination of transcription as well as translation. Bovine papilloma viral DNA vectors can be used to analyze, recover and stably express cloned human DNA sequences in heterologous cells.

Significance to Biomedical Research and the Program of the Institute:

Cellular morphology is maintained by the cytoskeletal organization and this organization is disrupted in neoplastic transformation. Actins are major components of the cytoskeleton that controls the cellular morphology. It is therefore important to study the basic structural components that make up the cytoskeleton and examine the possibility of their alteration in the tumor cells. Furthermore, the regulation of expression of genes involved in the make-up of the cytoskeletal structures and thus the morphology of the cell is essential to an understanding of the process of transformation. This project deals with the structure and regulation of actin genes and is highly relevant to the program of the institute. This work also provides information on the expression of cloned genes in heterologous cells and thus has implications in gene therapy.

Proposed Course:

Since the Section in which this work was planned is not operational at this time, further work on this project is suspended.

Publications:

Ueyama, H., Hamada, H., Battula, N. and Kakunaga, T.: Structure of a human smooth muscle actin gene (aortic type), with a unique intron site. Mol. Cell. Biol. 4: 1073-1078, 1984.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01CP04785-15 LMC

PERIOD COVERED
October 1, 1984 through September 30, 1985

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)
DNA Repair Studies on Human and Mouse Normal, Tumor, and Transformed Cells

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	R. S. Day, III	Research Physical Scientist	LMC	NCI
Others	T. Yagi	Visiting Fellow	LMC	NCI
	M. Babich	Staff Fellow	LMC	NCI
	C. A. Heilman	Senior Staff Fellow	LEC	NCI
	D. B. Yarosh	Cancer Expert	LMPH	NCI
	A. J. Fornace, Jr.	Cancer Expert	ROB	NCI

COOPERATING UNITS (if any)
Chemical Carcinogenesis Program, Litton Bionetics, Inc., Frederick, MD (D. Scudiero); Laboratory of Molecular Genetics, NICHD, NIH, Bethesda, MD (H. Okayama).

LAB/BRANCH
Laboratory of Molecular Carcinogenesis

SECTION
Nucleic Acids Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.2	2.2	0.0

CHECK APPROPRIATE BOX(ES)
☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The study of human cells defective in repairing damaged DNA was extended, with the rationale that DNA repair-deficient cells are more susceptible to the adverse effects of carcinogens (cell killing, mutagenesis, sister chromatid exchange, and malignant transformation) than their repair-proficient counterparts. A group of 19 human tumor and eight SV40-transformed strains almost totally deficient in the repair of O-6-methylguanine (O-6-MeG, a modified DNA base made by certain methylating agents) was identified earlier in this project. Such strains are called Mer-. Transformation by SV40, Rous sarcoma virus, adenovirus, or Epstein-Barr virus produces Mer- strains. Mer+ but not Mer- strains contain about 60,000 copies of a 22,000 MW O-6-MeG-DNA methyltransferase (O6DMT) that is responsible for repairing O-6-MeG by demethylation. Cell strains having intermediate amounts of O6DMT also have intermediate sensitivity to killing by 1-methyl-3-nitro-1-nitrosoguanidine (MNNG) or by 1-(2-chloroethyl)-1-nitrosourea (CNU), and to the induction of sister chromatid exchanges by MNNG. Human interferons α and β inactivate Mer- tumor strains while Mer+ human tumor strains are more resistant to such treatment, indicating an association between defective repair of O-6-MeG and sensitivity to interferons. Oncogene activation was linked to depletion of O6DMT in mouse cells by a study in which interferon was used to produce revertants that failed to express the human c-Ha-ras 1 gene. Compared to non-transformed cells or revertant cells that did not express the oncogene, transformed cells or revertant cells that showed oncogene expression had reduced O6DMT and post-CNU survival levels. A study done to investigate the role of poly ADP ribose polymerase in lethality due to O-6-MeG indicated no such association. However, poly ADP ribose polymerase is likely involved in the repair of 3- or 7-methylated adenines or guanines.

PHS 6040 (Rev. 1/84)

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

R. S. Day, III	Research Physical Scientist	LMC	NCI
T. Yagi	Visiting Fellow	LMC	NCI
M. Babich	Staff Fellow	LMC	NCI
D. B. Yarosh	Staff Fellow	LMC	NCI
C. A. Heilman	Senior Staff Fellow	LEC	NCI
A. J. Fornace Jr.	Cancer Expert	ROB	NCI

Objectives:

To learn more about DNA repair mechanisms in human and other mammalian cells and about their role in carcinogenesis. In particular, to determine the nature of the DNA repair defects both in human tumor cells and in cells from persons who are genetically predisposed to cancer. In addition, to use human cell strains with characterized defects to study the mechanisms of action of carcinogenesis or suspect carcinogens, chemotherapeutic agents, and other chemicals, in altering either DNA or the repair of damaged DNA.

Methods Employed:

1. Plaque assay: An adenovirus-host cell reactivation assay, developed previously in this project, was used to quantitate the deleterious effects of various chemical and physical treatments on the ability of the virus to initiate and sustain infection. The method involves establishing monolayer cell cultures which are infected with treated or non-treated adenovirus. The infected cells are then incubated 12-14 days with feeding by means of periodic overlaying with a nutrient agar. Non-treated virions or those treated ones which have been "reactivated" by cellular repair mechanisms form plaques of dead, lysed cells which are then counted.

2. Cellular extracts and partially purified fractions were assayed for O^6-methylguanine-DNA methyltransferase repair activity using as a substrate either DNA methylated by [^{3}H-methyl]-methylnitrosourea (MNU) or a synthetic double-stranded polymer (kindly supplied by Dr. S. Mitra, Oak Ridge National Laboratory) containing [^{3}H-guanine]-O^6-methylguanine. Reaction mixtures were incubated at 37°C, then heated in acid to remove purines, which were separated by high pressure liquid chromatography (HPLC) and quantified by liquid scintillation counting. A reduction of the O^6-methylguanine to guanine ratio or a conversion of O^6-methylguanine to guanine was interpreted to mean that repair of O^6-methylguanine had occurred.

3. The survival of cells treated with DNA-damaging chemicals was assayed by growth of the cells into colonies of at least 50 cells. When the inhibitor of poly ADP ribose polymerase, 3AB, was used, it was prepared in complete medium and used to treat cells for one hour before application of the DNA-damaging chemical to the cells.

4. Plasmid production and purification together with transfer to _E. coli_ and human cells, and assays for their presence followed published protocols.

5. The production and repair of methylated purines in DNA was followed using HPLC techniques, with either carbon-18 or strong cation exchange columns to separate both methylated bases and methylated deoxyribonucleosides.

Major Findings:

A major part of this year's research concerned the mechanisms involved in repair of DNA damage produced by alkylating agents (principally methylating agents), including chloroethylnitrosoureas (CNUs). Earlier in this project, we identified a group of 19 (of 93) human tumor cell strains that is unable to repair adenovirus damaged by MNNG. This repair-deficient phenotype we have termed Mer^-. Human tumor cells having the Mer phenotype fail to repair O^6-methylguanine (O^6MeG) lack a 22,000 MW protein called O^6MeG-DNA methyltransferase (O6DMT) and are extremely sensitive to MNNG in terms of both post-MNNG colony-forming ability and post-MNNG sister chromatid exchange (SCE) production. They fail to repair DNA:DNA cross links produced by chloroethylating nitrosoureas and are easily killed by these agents.

Normal appearing fibroblasts of two patients whose tumors gave rise to Mer^- strains were determined to be Mer^+. Moreover, Mer^- human tumor cells show more post-MNNG DNA repair synthesis but less post-MNNG semiconservative DNA synthesis than do human fibroblasts, and fail to restore control tertiary structure to their DNA after MNNG treatment.

We have also identified a group of five Mer^+ strains, that, while more resistant than Mer^- strains to MNNG in terms of post-treatment colony-forming ability, are about three-fold (slope difference) more MNNG-sensitive than are 13 human fibroblast strains or other Mer^+ strains. Rem^+, for resistance to MNNG, is the phenotype we have assigned to human fibroblasts, so that the MNNG-sensitive Mer^+ strains are Mer^+ Rem^-. Mer^+ Rem^- cells behave as if they contain about one-third of the O6DMT complement present in Mer^+ Rem^+ cells.

Mer^- strains are produced from Mer^+ strains by SV40 transformation, but these strains are Rem^+. Further, transformation by other viruses, adenovirus, Rous sarcoma, and Epstein-Barr virus, also produces Mer^- strains.

Thus, in human tumor lines, Mer function (i.e., O6DMT) may be shut off by oncogene activity or is produced in them after infection by an adventitious virus, also present in the tumor biopsy. We have identified two groups of human cells having the Mer^- Rem^+ phenotype, i.e., they fail to repair MNNG-damaged adenovirus (and fail to repair O^6MeG), but are resistant in terms of post-MNNG colony-forming ability as are normal human fibroblasts. The groups include four Mer cell strains obtained by SV40 transformation and a number of MNNG-resistant revertants of the A1235 Mer^- human astrocytoma cell strain. However, strains of the Mer^- Rem^+ phenotype are as sensitive as Mer^- Rem^- to inactivation by CNU or 3-hydroxyethyl-1-chloroethyl-1-nitrosourea (HECNU), believed to be lethal due to O^6-chloroethylguanine production and (if not repaired) consequent DNA:DNA cross-link formation. Thus the Mer^- phenotype correlates with lack of O^6MeG

repair activity, increased susceptibility to MNNG-produced SCEs, and sensitivity to killing by CNU or HECNU, but not with sensitivity to killing by MNNG.

We have shown that 6 strains of Mer^- human tumor cells are more sensitive to killing by human beta interferon (HuIFN-beta) than are 10 strains of Mer^+ human tumor strains. Similar data was obtained with HuIFN-alpha. Two Mer^- human tumor strains, which were converted to Mer^- after the establishment as Mer^+ in culture (one by Rous sarcoma virus transformation, the other by unknown causes), were resistant to the IFNs. Unlike MNNG treatment, HuIFN treatment did not produce O^6MeG in DNA and produced no sister chromatid exchanges in Mer^- cells. Our interpretation is that the HuIFNs inactivate genes responsible for the unrestricted growth in many Mer^- strains but do not in Mer^+ strains. During tumorigenesis, activation of growth promoting oncogenes in Mer^- cells would be causatively linked with a shutoff of Mer function. To test this hypothesis, we selected a mouse cell system in which a known oncogene is responsible for transformation and in which the oncogene is revertible by treatment with interferon. The transformed cells had deficient O6DMT, sensitivity to chloroethylnitrosourea (CNU), and an activated human c-Ha-ras 1 oncogene. We prepared three flat revertants using interferon: like the non-transformed NIH 3T3 cells, two had reduced transcription of the oncogene, elevated O6DMT, and sensitivity to CNU; the third, like the transformed RS 485 cells, had an activated oncogene, and indicators of reduced repair capacity. The data are strongly suggestive of a link between oncogene activation, repair of O^6MeG, and the mode of interferon action.

We have shown that the O6DMT which repairs O^6MeG in DNA also repairs the free base O^6MeG at about a million-fold lower rate. As a result, a two hour treatment of Mer^+ cells with 2mM O^6MeG (free base) reduces O6DMT activity because the protein acts stoichiometrically and is inactivated by its substrate. Mer^+ cells treated in this way are more sensitive to killing by CNU than are non-treated cells. The CNU sensitivity of Mer^- cells is little affected by the pretreatment, showing that repair cannot be blocked in a case in which little repair is possible, supporting our interpretation.

Inhibitors of poly ADP ribose polymerase are known to potentiate the effects of alkylating agents in killing certain rodent cell strains that failed to repair O^6MeG, but nevertheless showed elevated survival after MNNG treatment. These cells thus showed a phenotype similar to the Mer^- Rem^+ phenotype discovered in human cells and described earlier in this report. Because in initial work in our laboratory using 5-methylnicotinamide to block poly ADP ribose polymerase, little potentiation of MNNG-produced killing of human cells was observed, we hypothesized that the reason that inhibitors of poly ADP ribose polymerase were effective in potentiating killing of Mer^- Rem^+ strains was that such inhibitors convert them to Mer^- Rem^- strains, for instance by making O^6MeG lethal to the cells. We were able to test this hypothesis from several experimental angles. We believe the hypothesis to be incorrect because of the following results. 3-aminobenzamide (3AB, an inhibitor of poly ADP ribose polymerase) potentiates MNNG-killing of Mer^- Rem^- cells as well as Mer Rem^-, Mer^- Rem^+, and Mer^+ Rem^+ human strains. Killing by MMS (methyl methanesulfonate, a methylating agent that produces relatively little O^6MeG) is more greatly potentiated by 3AB than is killing by MNNG. 3AB likely enhances the lethal effects of other major methylated bases such as 3-methyladenine or 7-methylguanine.

Significance to Biomedical Research and the Program of the Institute:

The results of this project suggest the possibility that a fraction, one-fifth, of all human tumors is composed of repair-defective cells. Certain bifunctional alkylating agents 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU), 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea (CCNU), are known to be effective against some individual human tumors and to be relatively ineffective against others, also indicating the possibility that the molecular basis for the success of alkylation chemotherapy may be specific to a tumor or a group of tumors. Our studies of the mechanisms by which repair occurs and of the inhibition of such repair are designed, in part, to understand ways by which tumors might be more successfully treated and ways by which tumors might arise. Physical, chemical, and viral carcinogens are all known to alter the structural integrity of the cellular genetic apparatus. An evaluation of the role of DNA repair and/or related mechanisms in conferring resistance or susceptibility to mutagenesis and carcinogenesis is an important facet in any overall program having as its goal the understanding of the molecular pathways which, when perturbed, give rise to carcinogenesis in humans. It is the long-range goal of this project to determine whether or not the elucidation of repair mechanisms is important to the understanding of carcinogenesis. It is expected that an understanding of human repair mechanisms, in general, will benefit many areas of biomedical research.

Proposed Course:

1) To continue surveying human strains for Mer^- strains using the MNNG-treated adenovirus plaque assay and the O6DMT activity assay; 2) to clone the O^6-methyltransferase gene; 3) to purify the O6DMT from human tissue, including placenta and liver; and 4) to pursue the relationship of interferon sensitivity to the Mer^- condition.

Publications:

Doniger, J., Day, R. S., III and DiPaolo, J. A.: Quantitative assessment of the role of O^6-methylguanine in the initiation of carcinogenesis by methylating agents. Proc. Natl. Acad. Sci. USA 82: 421-425, 1985.

Scudiero, D. A., Meyer, S. A., Clatterbuck, B. E., Mattern, M. R., Ziolkowski, C. H. J. and Day, R. S., III: Sensitivity of human cell strains having different abilities to repair O^6-methylguanine in DNA to inactivation by alkylation agents including chloroethylnitrosoureas. Cancer Res. 44: 2467-2474, 1984.

Yarosh, D. B., Scudiero, D. A., Yagi, T. and Day, R. S. III: Human tumor cells strains both unable to repair O^6-methylguanine and hypersensitive to killing by human alpha and beta interferons. Carcinogenesis. (In Press)

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05086-07 LMC

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Monoclonal Antibodies to Cytochrome P-450 from Human Tissue

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	S. S. Park	Senior Staff Fellow	LMC	NCI
Others:	H. V. Gelboin	Chief	LMC	NCI
	H. Miller	Biological Lab Technician	LMC	NCI

COOPERATING UNITS (if any)

University of Oulu, Finland (O. Pelkonen)

LAB/BRANCH

Laboratory of Molecular Carcinogenesis

SECTION

Metabolic Control Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.0	0.5	0.5

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Xenobiotics such as drugs and carcinogens as well as endobiotics such as steroids and fatty acids are metabolized by the mixed function oxidase systems. Cytochrome P-450 is the key component of mixed function oxidases and the type and quantity of specific forms of cytochrome P-450 determine the disposition of a particular substrate. MAbs are specific probes for particular antigenic determinants and are useful tools for identification of particular isoenzymes. Myeloma cells were hybridized with spleen cells of mice immunized with purified human placenta mitochondorial cytochrome P-450. We obtained 25 independent hybridomas producing MAbs to the human placenta mitochondrial cytochrome P-450. Placenta microsomal and mitochondrial cytochromes P-450 play important roles in steroid metabolism. In addition 3-methylcholanthrene inducible cytochromes P-450 of rats are also inducible in human placenta by smoking. Since the patterns of cytochromes P-450 present in human liver, placenta and other organs can be phenotyped with specific MAbs, the roles and interaction of different forms of cytochromes P-450 will be examined as possible markers of individaul differences in drug metabolism and sensitivity to environmental carcinogens.

PHS 6040 (Rev. 1/84)

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

S. S. Park	Senior Staff Fellow	LMC	NCI
H. V. Gelboin	Chief	LMC	NCI
H. Miller	Biological Lab Technician	LMC	NCI

Objectives:

Xenobiotic drugs, carcinogens and endobiotics such as steroids and fatty acids are metabolized by mixed function oxidase systems. Cytochrome P-450 is the key component and many different forms of cytochrome P-450 have been reported. Specific forms appear to predominate following treatment with specific inducers. Specific carcinogen-metabolizing enzymes might predominate in individuals who smoke heavily, or who are exposed to carcinogenic environmental chemicals. Microsomal preparations of placenta from women who smoke heavily possess high levels of mixed function oxidases, as do human monocytes, lymphocytes, and lung cell lines induced with benzo(a)anthracene. The objective of this work is to prepare monoclonal antibodies (MAbs) to cytochrome P-450 of human placenta mitochondria and characterize the specificities of the MAbs for different forms of cytochrome P-450. The use of MAbs may help to define individual differences in drug and carcinogen metabolism.

Methods Employed:

Balb/c female mice were immunized with cytochrome P-450 derived from human placenta mitochondria. The primed spleen cells were isolated and fused with myeloma cells using polyethylene glycol. The hybrid cells are grown in a selective medium (HAT) and screened by radioimmunoassay for hybridomas producing MAbs to the immunogen. MAb-producing hybridomas were cloned by dispensing cell suspensions with the probability of having 0.4 cell to each well of microtiter plates. Three successive clonings were done to insure the monoclonality of the hybridoma line.

Major Findings:

Twenty five independent hybrid clones were obtained from two hybridizations between mouse myeloma cells and spleen cells derived from mice immunized with cytochrome P-450 of human placenta mitochondria. The binding affinity of the MAbs was 20-30 times greater than the nonspecific MAbs in initial screening but the affinity of binding decreased often repeated use of antigens in the solid phase RIA, probably because of the unstable nature of human cytochrome P-450. We also found that spleen cells frozen by programmed freezing are as viable as fresh cells in hybridization. This result suggests that we can use spleen cells transported over long distance, especially human spleen cells which are not easily available. The MAbs to human placenta mitochondial cytochrome P-450 will be characterized with respect to cross-reactivity and enzyme inhibition.

Significance to Biomedical Research and the Program of the Institute:

Placenta microsomal and mitochondorial cytochrome P-450 play important roles in sterioid transformation during pregnancy. We have also found that 3-methylcholanthrene inducible cytochrome P-450 is inducible by smoking. Since MAbs are specific probes for phenotyping and identification of cytochrome P-450 isozymes, a series of MAbs to each isozyme can help to identify the relationship of different forms of cytochrome P-450 and individual differences in the metabolism of steroids and other drugs. The finding that frozen spleen cells are as capable as fresh cells in hybridization and production of MAb cells indicates that we can use spleen from different parts of the world.

Proposed Course:

1) Additional MAbs to placenta microsomal cytochromes P-450 and other isozymes from human sources will be prepared. 2) MAb-directed competitive radioimmunoassays and Western blot techiques will be utilized to identify known as well as unknown cytochromes P-450 from different human sources. 3) The cross-reactivity of MAbs to human enzymes with iso-enzymes from different species will be studied.

Publications:

Friedman, F. K., Robinson, R. C., Song, B. J., Park, S. S., Crespi, C. L., Marletta, M. A. and Gelboin, H. V.: Monoclonal antibody-directed determination of cytochrome P-450 types expressed in a human lymphoblastoid cell line. Mol. Pharmacol. 27: 652-655, 1985.

Fujino, T., Gottlieb, K., Manchester, D. K., Park, S. S., West, D., Gurtoo, H. L. and Gelboin, H. V.: Monoclonal antibody phenotyping of inter-individual differences in cytochrome P-450 dependent reactions of single and twin human placenta. Cancer Res. 44: 3196-3923, 1984.

Fujino, T., West, D., Park, S. S. and Gelboin, H. V.: Monoclonal antibody directed aryl hydrocarbon hydroxylase and 7-ethoxycoumarin deethylase in mammalian tissues. J. Biol. Chem. 259: 9044-9050, 1984.

Gelboin, H. V., Fujino, T., Song, B. J., Park, S. S., West D., Robinson, R., Miller, H. and Friedman, F. K.: Monoclonal antibody directed phenotyping of cytochrome P-450 by enzyme inhibition, immunopurification and radioimmunoassay. In Omenn, G. and Gelboin, H. V. (Eds.): Banbury Report 16, Genetic Variability in Responses to Chemical Exposure. New York, Cold Spring Harbor Laboratory, 1984, pp. 65-84.

Song, B. J., Friedman, F. K., Park, S. S., Tsokos, G. C. and Gelboin, H. V.: Monoclonal antibody-directed radioimmunoassay detects cytochrome P-450 in human placenta and lymphocytes. Science 228: 490-492, 1985.

Song, B. J., Fujino, T., Park, S. S., Friedman, F. K. and Gelboin, H. V.: Radioimmunoassay of monoclonal antibody-specific cytochrome P-450. _J. Biol. Chem._ 259: 1394-1397, 1984.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05109-06 LMC

PERIOD COVERED

October, 1984 to September 30, 1985

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Chromatin Structure and Carcinogenesis

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	Michael Bustin	Acting Section Head	LMC	NCI
Others:	Leo Einck	Senior Staff Fellow	LMC	NCI
	Thyagarajan Srikantha	Visiting Fellow	LMC	NCI
	Nirmolini Soares	Laboratory Tech. (Microbiol.)	LMC	NCI

COOPERATING UNITS (if any)

Department of Cell Biology, New York University School of Medicine, New York, N.Y. (Dr. Adesnick)

LAB/BRANCH

Laboratory of Molecular Carcinogenesis

SECTION

Protein Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.7	2.2	0.5

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The chromatin structure of genes coding for P-450 enzymes is investigated. Changes in the chromatin structure of these genes upon gene activation is examined by comparing the chromatin structure of the genes in nuclei purified from the livers of both normal and carcinogen-treated rats. Micrococcal nuclease digestion reveals that one of the genes, P-450M, is present in a non-nucleosomal conformation. Four DNase I hypersensitive sites have been mapped in this gene. The sites do not change upon gene induction and are not present in tissues, such as rat thymus, which do not express this gene. The P-450M gene seems to be associated with the nuclear matrix. Using a plasmid containing the entire cloned P-450M sequence, an S1 sensitive site has been mapped. The DNA region recognizable by S1 may also be recognized by regulatory chromosomal proteins.

PHS 6040 (Rev. 1/84)

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged in this Project:

Michael Bustin	Acting Chief	LMC	NCI
Leo Einck	Senior Staff Fellow	LMC	NCI
Thyagarajan Srikantha	Visiting Fellow	LMC	NCI
Nirmolini Soares	Laboratory Tech. (Microbiol.)	LMC	NCI

Objectives:

To study the molecular mechanisms involved in the activation of carcinogen metabolizing enzymes by examining genomic changes associated with exposure of organisms to chemical carcinogens.

Methods Employed:

Exposure of organisms to a variety of xenobiotics and drugs induces the synthesis of enzymes belonging to the P-450 family of mixed function oxidases. Induction of gene expression often is correlated with detectable changes in the chromatin structure of the inducible genes. Elucidation of the chromatin structure of the enzymes involved in the metabolism of drugs and carcinogens may help elucidate the molecular mechanisms involved in carcinogenesis. We have concentrated our efforts on studying the chromatin structure of two genes inducible by 3-methylcholanthrene and genes inducible by phenobarbital. Genomic clones are subcloned in pBR322 to give probes which can be radioactively labelled by nick-translation or primer extension and used in Southern blots to analyze the structure of the gene. Nuclei are isolated from the liver of control and carcinogen-treated rats, the DNA isolated, restricted, and the restriction fragments separated on agarose gels and transferred to a solid support.

Digestion of nuclei with micrococcal nuclease and examination of the DNA isolated from such nuclei allows determination of the nucleosomal structure of the gene. Examination of the Southern hybridization pattern with DNA isolated from DNase I digested nuclei allows mapping of the DNase I sensitive sites in the gene. The structure of both the inducible and noninducible P-450 genes from both inducible and noninducible tissues can be examined. S1 sensitive sites are mapped in plasmids containing various subclones of the gene. The plasmids are cut with S1 under various conditions, the digested plasmid restricted and the S1 site mapped by Southern analysis with known probes.

Major Findings:

The chromatin structure of 3 types of genes was investigated. Gene P-450M codes for a constitutive P-450 enzyme whose synthesis is induced 4-fold upon treatment of rats with 3-MC. Gene P-450c codes for an enzyme whose synthesis is induced over 100-fold by the same treatment. Genes P-450b and P-450e code for phenobarbital inducible cytochromes P-450.

The P-450M gene has been subcloned into pBR322 to give a set of 14 subclones overlapping the entire gene. Using these subclones, we found that:

1. The coding regions of the P-450M gene are more sensitive to DNase I digestion than bulk nuclear DNA or uninduced genes.

2. The gene is not present in the typical 200 bp nucleosomal structure characteristic of bulk DNA.

3. Four DNase I hypersensitive sites are present in the 5' terminal region of the gene.

4. An S1 nuclease sensitive site is located close to a DNase I hypersensitive site. The location of this site, and the DNase I hypersensitive sites are indicated in the diagram presented in Fig. 1 below:

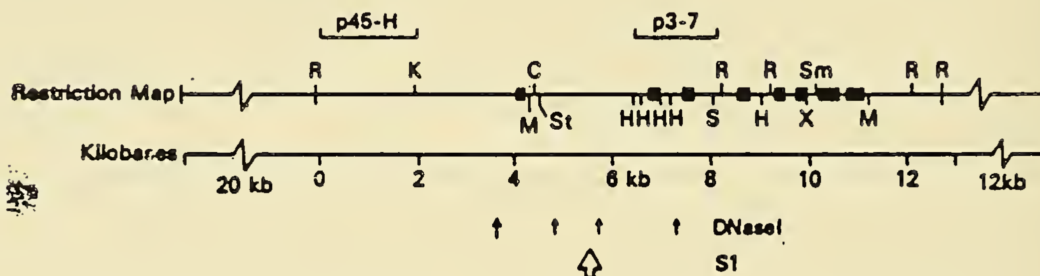

Fig. 1. Location of the DNase I hypersensitive sites and the major S1 cleavage site in the P-450M gene. The 12.4 kb genomic fragment contains the entire coding region of the P-450M gene flanked by 4 kb upstream and 2 kb downstream untranslated sequences. The exons are marked by boxes. The letters indicate various restriction sites.

5. Gene induction by treatment with 3-methylcholanthrene does not change the DNase hypersensitive sites.

6. Rat thymus does not contain these sites suggesting that in the liver the chromatin structure is altered to allow tissue-specific expression of the gene.

Probes which will allow investigation of the chromatin structure of P-450c and P-450e are currently subcloned into pBR322 and examined for their suitability for studies on the chromatin structure of these genes.

Significance to Biomedical Research and the Program of the Institute:

Elucidation of the molecular processes involved in the induction of enzymes which metabolize xenobiotics and drugs may aid in understanding basic mechanisms involved in determining individual differences in drug toxicity and carcinogen sensitivity. Regulation of the expression of these enzymes may be dictated by the chromatin structure of the genes coding for the enzymes. Studies on the chromatin structure of P-450 genes will help in elucidating certain aspects of the manner in which cells respond to carcinogens and mutagens and ultimately may help in isolating and studying molecules which regulate the expression of these genes.

Proposed Course:

The project represents an in-depth and comprehensive study of the molecular mechanisms involved in regulating the expression of genes which code for enzymes whose activities are important for metabolizing carcinogens, mutagens and other xenobiotics.

The chromatin structure of several other enzymes belonging to the P-450 gene family will be determined. In the next year, the efforts will be concentrated on studying the chromatin structure of the P-450c and P-450e genes. Once these are established, we will study the association of these genes with subcellular compartments, such as the nuclear matrix, and search for proteins or other macromolecules which regulate the chromatin structure of these genes. The techniques and approaches developed will be useful for investigating the regulation of the human P-450 genes.

Publications:

Einck, L., Fagan, J., and Bustin, M.: The chromatin structure of a 3-methyl cholanthrene induced P-450 gene. Biochemistry. (In Press)

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05125-05 LMC

PERIOD COVERED
October 1, 1984 to September 30, 1985

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)
Preparation of Monoclonal Antibodies to Cytochromes P-450

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	S. S. Park	Senior Staff Fellow	LMC	NCI
Others:	H. V. Gelboin	Chief	LMC	NCI
	H. Miller	Biological Lab. Technician	LMC	NCI

COOPERATING UNITS (if any) Vanderbilt Univ. School of Med., Nashville, TN (F. P. Guengerich); Univ. of Connecticut, Farmington, CT (J. B. Schenkman); Woods Hole Oceanographic Institute, Woods Hole, MA (J. Stegeman); PA State Univ., Hershey, PA (E. S. Vesell); Laboratory of Chemical Pharmacology, NHLBI, NIH (J. R. Gillette)

LAB/BRANCH
Laboratory of Molecular Carcinogenesis

SECTION
Metabolic Control Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.0	0.5	0.5

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Most chemical carcinogens are activated to ultimate carcinogens. Polyaromatic hydrocarbons, such as benzo(a)pyrene, are activated by mixed-function oxidases, the key components of which are cytochromes P-450. Identification and quantitation of cytochrome P-450 isozymes are essential to understanding the role of cytochrome P-450 in the primary process of chemical carcinogenesis and individual differences in sensitivity to chemical carcinogens. Our approach is to prepare monoclonal antibodies (MAbs) specific for individual and classes of cytochrome P-450. In addition to the MAbs directed to cytochromes P-450 of rats treated with phenobarbital (PB-P-450), 3-methylcholanthrene (MC-P-450), and β-naphthoflavone (BNF-P-450), we prepared and characterized MAbs to pregnenolone 16-α- carbonitrile induced PCN-P-450 and environmentally induced marine fish cytochrome P-450. Among 11 MAbs to PCN-P-450, 8 were specific to PCN-P-450 and 3 cross-reacted with PB-P-450E. We also prepared MAbs to uninduced forms of cytochrome P-450. Among 9 MAbs to Scup-P-450, 8 were specific to Scup-P-450 and one recognized both Scup-P-450 and rat liver MC-P-450. MAbs to uninduced forms of cytochrome P-450 were all IgM types. These MAbs have been utilized to phenotype and purify cytochromes P-450 from tissues and organs of animals and humans.

Z01CP05125-05 LMC

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

S. S. Park	Senior Staff Fellow	LMC	NCI
H. V. Gelboin	Chief	LMC	NCI
H. Miller	Biological Lab. Technician	LMC	NCI

Objectives:

The prototype environmental chemical carcinogen, benzo(a)pyrene (BP), is activated by aryl hydrocarbon hydroxlase (AHH) and epoxide hydrolase, both of which exist in multiple forms. Metabolism of BP by these enzymes leads to detoxification as well as carcinogenesis. Exposure of individuals to inducers also influences metabolism through induction of specific isozymes. The primary objective is to build a large library of MAbs to the various isozymes of carcinogen and drug metabolism. We have started with the cytochromes P-450 and presently have successfully prepared panels of more than 200 MAbs to nine different cytochromes P-450.

Methods Employed:

Balb/c female mice were immunized with purified cytochrome P-450 of rats which were untreated or treated with inducers. The primed spleen cells were isolated and fused with myeloma cells, using polyethylene glycol. The hybrid cells were grown in a selective medium (HAT) and screened by radioimmunoassay (RIA) to identify hybridomas producing MAbs to constitutive forms of cytochrome P-450, PCN-P-450 or Scup-P-450. The effects of MAbs on cytochrome P-450 catalytic and binding activities were measured. Ouchterlony double immunodiffusion analysis and competitive radioimmunoassay were also carried out for the characterization of the MAbs.

Major Findings

1) Eleven hybridomas producing MAbs to PCN-P-450 were obtained: eight were IgG1, one IgG2 and two were IgG2b. Based on the interaction with PCN P-450 and PB-P-450, these MAbs can be classified into two groups: binding to only PCN-P-450 and binding to both PCN P-450 and PB-P-450. These MAbs did not inhibit the catalytic activities of PCN P-450 for BP, 7-ethoxycoumarin, ethylmorphine and benzphetamine metabolism. All the MAbs gave precipitin reactions with PCN P-450 upon prolonged incubation at room temperature. 2) Nine hybridomas produced MAbs specific to Scup P-450 except one which also recognized MC-P-450. One of the 9 MAbs were precipitating and the inhibition of catalytic activities varied. All MAbs to constitutive forms of cytochrome P-450 were IgM types.

Significance to Biomedical Research and the Program of the Institute:

The balance of BP metabolism between detoxification and carcinogen formation is dependent on the types and amounts of isozymes present. Each isozyme of cytochrome P-450 possesses specific antigenic determinants (epitopes) and the MAbs prepared to them are epitope-specific. Antigenically related cytochromes P-450 can be induced by different chemicals. MAbs prepared to rat or rabbit enzymes are also cross-reactive with human cytochrome P-450. They are useful in phenotyping isozyme patterns of individuals and relating these to individual differences in carcinogen sensitivity. MAbs to the environmentally induced marine fish, Scup-P-450, bound to MC-P-450 and BNF-P-450 and would be useful for monitoring environmental pollution and for phylogenetic studies. The MAbs to uninduced and induced forms of cytochromes P-450 are also very important in the study of interactions which determine the direction of carcinogen metabolism in individuals exposed to different environments. Our MAbs have also been very useful tools for immunopurification, detection, and reaction phenotyping of cytochromes P-450.

Proposed Course:

We plan to prepare additional MAbs using a variety of P-450 preparations from rats and other animals which are either induced or untreated. These additional probes for different cytochromes P-450 will make feasible more extensive characterization and phylogenetic studies of the isozymes in various sources including human tissues.

Publications:

Cheng, K. C., Friedman, F. K., Song, B. J., Park, S. S., and Gelboin, H. V.: Detection and purification of cytochromes P-450 in animal tissues with monoclonal antibodies. J. Biol. Chem. 259: 12279-12284, 1984.

Cheng, K., Krutzsch, H. C., Park, S. S. Grantham, P. H., Gelboin, H. V. and Friedman, F. K.: Amino-terminal sequence analysis of six cytochrome P-450 isozymes purified by monoclonal antibody directed immunopurification. Biochem. Biophys. Res. Commun. 123: 1201-1208, 1984.

Friedman, F. K., Park, S. S., Fujino, T., Song, B. J., Robinson, R. C., West, D., Radkowsky, A. K., Miller, H. and Gelboin, H. V.: Phenotyping cytochromes P-450 with monoclonal antibodies. Toxicol. Pathol. 12: 155-161, 1984.

Friedman, F. K., Robinson, R. C., Song, B. J., Park, S. S., Crespi, C. L., Marletta, M. A. and Gelboin, H. V.: Monoclonal antibody-directed analysis of cytochrome P-450 expression in a human lymphoblastoid cell line. Mol. Pharmacol. (In Press)

Gelboin, H. V., Park, S. S., Fujino, T., Song, B. J., Cheng, K. C., Miller, H., Robinson, R., West, D. and Friedman, F. K.: Cytochrome P-450, xenobiotic and endobiotic metabolism: Monoclonal antibody directed detected, purificaton and reaction phenotyping. In Boobis, A. R., Caldwell, J., deMatteis F. and Elcombe, C. R. (Eds.): Microscomes and Drug Oxidations. London, Taylor and Francis, 1985, pp 390-401.

Hietanen, E., Bartsch, H., Ahotupa, M., Park, S. S. and Gelboin, H. V.: Tissue specificity of extrahepatic monooxygenase in the metabolism of xenobiotics. Proc. IX Eur. Drug Metabol. Workshop. (In Press)

Park, S. S, Fagan, J. B., Fujino, T., Friedman, F., Song, B. J., Park, K. H., Miller, H., West, D., Pastewka, J., Robinson, R. and Gelboin, H. V.: Monoclonal antibodies in identification of mRNA trans lation products and cloned cDNA. In Han, M. H. (Ed.): Symp. on Genetic Eng. and Biotech. Seoul, Korea, KAIST. (In Press)

Park, S. S., Fujino, T. and Gelboin, H. V.: Monoclonal antibody-directed phenotyping for cytochrome P-450 in animals and humans. In Chuo, W. K. (Ed): 9th Symp. on Science and Technol. Seoul, Fed. Korean Sci. Eng. Assoc., 1984, pp. 760-764.

Park, S. S., Fujino, T., Miller, H., Guengerich, F. P., and Gelboin, H. V.: Monoclonal antibodies to phenobarbital induced rat liver cytochrome P-450. Biochem. Pharmacol. 33: 2071-2081, 1984.

Snider, M. D., Brands, R., Hino, Y., Gelboin, H. V., Park, S. S. and Rothman, J. E.: The retention of membrane proteins by the endoplasmic reticulum. J. Cell Biol. (In Press)

Song, B. J., Friedman, F. K., Cheng, K. C., Park, S. S. and Gelboin, H. V.: Monoclonal antibody-directed radioimmunoassay and immunopurification of cytochrome P-450. In Chuo, W. K. Symp. on Science and Technol. Seoul, Fed. Korean Sci. Eng. Assoc., 1984, pp. 804-810.

Song, B. J., Friedman, F. K., Park, S. S., Tsokos, G. C. and Gelboin, H. V.: Monoclonal antibody-directed radioimmunoassay detects cytochrome P-450 in human placenta and lymphocytes. Science 228: 490-492, 1985.

Wiebel, F. J., Park, S. S., Kiefer, F. and Gelboin, H. V.: Expression of cytochromes P-450 in rat hepatoma cells: Analysis by monoclonal antibodies specific for 3-methylcholanthrene or phenobarbital-induced rat liver cytochrome P-450. Eur. J. Biochem. 145: 455-462, 1984.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05196-05 LMC

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Cloning and Characterization of Human Cytochrome P-450

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	Frank J. Gonzalez	Senior Staff Fellow	LMC	NCI
Others:	Harry V. Gelboin	Chief	LMC	NCI
	Byung J. Song	Visiting Fellow	LMC	NCI
	Jullia Pastewka	Chemist	LMC	NCI
	Anil. K. Jaiswal	Visiting Fellow	LDP	NICHD
	Daniel W. Nebert	Chief	LDP	NICHD

COOPERATING UNITS (if any)

Argonne National Laboratory, Argonne, IL (J.P. Hardwick)

LAB/BRANCH

Laboratory of Molecular Carcinogenesis

SECTION

Metabolic Control Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.0	1.0	1.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Immunochemical methods are being employed to isolate human cytochrome P-450 genes. Cytochrome P-450s are purified from rat liver and extensively characterized with regard to inducibility by xenobiotics and steroids, and catalytic activity. Rabbit anti-cytochrome P-450 is then produced and utilized to screen human cDNA expression libraries. Full length cDNAs are isolated and their sequences are determined. These cDNAs are compared with their rodent P-450 counterparts. After sufficient characterization, P-450 cDNA probes will be used to analyze the regulation of cytochrome P-450 in cultured human cells and lymphocytes. In addition, these probes will be utilized to examine human genetic differences. As a model system we will first attempt to characterize the gene coding for the human debrisoquine 4-hydroxylase. The polymorphism of this enzyme system in humans has been well documented.

PHS 6040 (Rev. 1/84)

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

Frank J. Gonzalez	Senior Staff Fellow	LMC	NCI
Jullia Pastewka	Chemist	LMC	NCI
Byung J. Song	Visiting Fellow	LMC	NCI
Harry V. Gelboin	Chief	LMC	NCI
Daniel W. Nebert	Chief	LDP	NICHD
Anil K. Jaiswal	Visiting Fellow	LDP	NICHD

Objectives:

1) Clone and characterize human P-450 cDNAs.
2) Clone and characterize human P-450 genes.
3) Study the regulation of human P-450s and compare with rodent systems.
4) Examine human genetic polymorphisms in P-450s with particular emphasis on debrisoquine 4-hydroxylase and putative polymorphisms in carcinogen metabolism.

Methods Employed:

Human cytochrome P-450s will be isolated by immunochemical techniques from cDNA expression libraries. These will be constructed by use of the Young and Davis vector Xgt11, by standard protocols. DNA sequencing will be carried out by use of m13 cloning and dideoxy sequencing. Computer analysis and comparisons will be made using programs available at the NIH. DNA isolation, RNA isolation, Southern blots, Northern blots and mRNA quantitation will be performed.

Major Findings:

Debrisoquine 4-hydroxylase was purified from rat liver microsomes by Dr. James Hardwick at Argonne National Laboratory, Argonne, Illinois. Polyclonal antiserum was prepared in rabbits and utilized to screen rat liver and human liver Xgt11 cDNA expression libraries.

Phage DNA was purified and the clones with the largest cDNA inserts were processed further. The inserts from the phage were subcloned into pBR322. Large amounts of plasmid DNA was purified and inserts isolated and processed for shotgun sequencing by use of the m13 cloning-dideoxy sequencing strategy. Sequence is analyzed with the Staden consensus program and assorted sequence analysis programs available at NIH.

Significance to Biomedical Research and the Program of the Institute:

Cytochrome P-450s are the major enzymes responsible for the metabolism of foreign chemicals. These include therapeutic drugs in addition to toxic and carcinogenic environmental and industrial chemicals. It has long been known that individuals

vary in their susceptibility to drugs and chemical carcinogens (industrial epidemiology of chemical exposure). Variations in the complex gene superfamily of cytochrome P-450s may account for some of this variability. These questions are being addressed in our laboratory at the level of direct genetic analysis. The debrisoquine 4-hydroxylase deficiency will serve as a model to begin to analyze defective cytochrome P-450 genes.

Proposed Course:

1) Sequence and compare rat and human debrisoquine 4-hydroxylase full length cDNAs.

2) Characterize the rat and human debrisoquine 4-hydroxylase gene.

3) Compare points 1 and 2 with other families of P-450 genes.

4) Analyze the defect in certain individuals in the debrisoquine 4-hydroxylase by restriction fragment lengths polymorphisms or by cloning and sequencing the normal and defective human gene.

Publications:

Jaiswal, A. K., Gonzalez, F. J. and Nebert, D. W.: Human dioxin-inducible cytochrome P-450: Complementary DNA and amino acid sequence. Science 228: 80-83, 1985.

Jaiswal, A. K., Gonzalez, F. J. and Nebert, D. W.: Human P-450 gene sequence and correlation of mRNA with genetic differences in benzo(a)pyrene metabolism. Nucl. Acids Res. (In Press)

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05204-05 LMC

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Carcinogen-induced Mutagenesis of a Shuttle Vector Plasmid in Human Cells

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Michael Seidman	Expert	LMC NCI
Others:	Anders Bredberg	Visiting Fellow	LMC NCI
	Miroslava Protic-Sabljic	Visiting Fellow	LMC NCI
	Kenneth Kraemer	Senior Surgeon	LMC NCI

COOPERATING UNITS *(if any)*

None

LAB/BRANCH

Laboratory of Molecular Carcinogenesis

SECTION

Protein Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.0	1.0	1.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

A shuttle vector plasmid was constructed which carries a small marker gene and which replicates in human and in bacterial cells. Plasmids treated with ultraviolet light were introduced into repair-proficient and repair-deficient human cells. Plasmids with UV-induced mutation in the marker gene were identified and characterized in a sensitive microbiological assay. The precise nature of the mutations was determined by DNA sequence analysis. The nature and location of changes were different in the repair-proficient and -deficient cells. This is the first molecular determination of carcinogen-induced mutations in human cells.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

Michael Seidman	Expert	LMC	NCI
Anders Bredberg	Visiting Fellow	LMC	NCI
Miroslava Protic-Sabljic	Visiting Fellow	LMC	NCI
Kenneth Kraemer	Senior Surgeon	LMC	NCI

Objectives:

To understand the relationship between carcinogen induced mutations in human cells and oncogene activation in human tumors.

Methods Employed:

A shuttle vector plasmid system was employed for mutagenesis studies. The nature of sequence changes was determined by DNA sequence analysis.

Major Finding

Shuttle vector plasmids carrying bacterial marker genes would appear to be powerful tools for studying DNA repair and mutagenesis in human and other mammalian cells. These plasmids can replicate in both bacteria and mammalian cells. Ultraviolet treated plasmid can be passaged through mammalian cells during which time, replication, repair, and mutagenesis will occur. The plasmid population can then be recovered and introduced into bacteria for assay. Bacterial colonies with plasmids containing mutations in the marker gene can be identified and quantified using sensitive microbiological assays. However, this approach is not in general use in part because of the results of recent experiments which demonstrated an unexpectedly high level of spontaneous mutagenesis when the plasmids were passaged through mammalian cells. The majority of the mutant plasmids carried deletions which arose following the introduction of double strand breaks into the plasmids early in the transfection. Point mutations also occurred. This high spontaneous mutation rate (about 1% in our initial experiments) made difficult the unambiguous recognition and characterization of mutations induced by DNA damaging agents.

Recently, we constructed a shuttle vector plasmid designed to reduce the frequency of spontaneous mutant plasmids which appear in the bacterial screening step. This plasmid (pZ189) contains a small marker gene, coding for a suppressor tRNA, which is placed between sequences necessary for plasmid replication and maintenance in bacteria. Consequently, many large deletions which would affect the tRNA gene would also preclude survival in bacteria. We find that the spontaneous mutation frequency of this marker in repair proficient human cells is about 0.05%, while in a xeroderma pigmentosum complementation group A cell line, the spontaneous mutation frequency is about 0.01%. We treated pZ189 with ultraviolet radiation and then passaged the damaged plasmid through the two cell

lines and into indicator bacteria. As expected, survival of the plasmid replicated in the xeroderma pigmentosum cells was significantly reduced relative to that in the normal cells at the same ultraviolet dose. The frequency of mutant plasmids recovered from both cell lines increased in a dose dependent manner. Treatment of the plasmid with 100 J/m^2 gave a mutation frequency of 0.25% in the XP cells. Plasmid treatment with 1000 J/m^2 gave a mutation frequency of about 1.5% in the normal human cells. We have determined the sequence of the altered tRNA genes in a number of mutant plasmids from both cell lines. In addition, we have determined the sites of ultraviolet damage in the marker gene. We find that the principal mutational change in the XP cells is the G:C to A:T transition which occurs at only a few of the sites of ultraviolet damage. The sequence changes in the normal cells are much more varied and include transversions as well as transitions and are located at many more of the sites of the original ultraviolet damage. This shuttle vector plasmid system should be useful for studying mutagenesis induced by many DNA damaging agents in a variety of mammalian cell lines.

Significance to Biomedical Research and the Program of the Institute:

Individuals with xeroderma pigmentosum suffer a high incidence of ultraviolet induced cancers. Our mutagenesis data lay the foundation for understanding the relationship between UV-induced mutagenesis and UV-induced oncogene activation.

Proposed Course:

Dr. Kraemer will continue the studies of the spectrum and location of carcinogen-induced mutation in cells from individuals with a variety of genetic dispositions to cancer. These will include additional xeroderma complementation groups.

Publications:

Razzaque, A., Chakrabarti, S., Joffe, S. and Seidman, M.: Mutagenesis of a shuttle vector plasmid in mammalian cells. Mol. Cell. Biol. 4: 435-441, 1984.

Razzaque, A., Mizusawa, H. and Seidman, M: Rearrangement and mutagenesis of a shuttle vector plasmid after passage in mammalian cells. Proc. Natl. Acad. Sci. USA 80: 3010-3014, 1983.

Seidman, M. M., Dixon, K., Razzaque, A., Zagursky, R. and Berman, M. L.: A shuttle vector plasmid for studying carcinogen induced mutations in mammalian cells. Gene. (In Press)

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05205-04 LMC

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT (*80 characters or less. Title must fit on one line between the borders.*)

Naturally Occurring Inhibitors of Different Forms of Cytochrome P-450

PRINCIPAL INVESTIGATOR (*List other professional personnel below the Principal Investigator.*) (*Name, title, laboratory, and institute affiliation*)

P. I.	H. V. Gelboin	Chief	LMC	NCI
Others:	F. K. Friedman	Senior Staff Fellow	LMC	NCI

COOPERATING UNITS (*if any*)

T. Sugimura, National Cancer Institute, Tokyo, Japan
P. M. Dewick, University of Nottingham, England

LAB/BRANCH

Laboratory of Molecular Carcinogenesis

SECTION

Metabolic Control Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.2	0.1	0.1

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects
☐ (a1) Minors
☐ (a2) Interviews

☐ (b) Human tissues

☒ (c) Neither

SUMMARY OF WORK (*Use standard unreduced type. Do not exceed the space provided.*)

The cytochromes P-450 metabolize a wide variety of drugs, chemicals and carcinogens. Specific modulators of aryl hydrocarbon hydroxylase (AHH) are useful probes for the study of the multiplicity, diversity, and different catalytic properties of the cytochromes P-450. We have examined the effect of a number of flavones on catalytic activity of rat hepatic microsomes. The compounds may be classified according to their activating or inhibitory effect on the constitutive and 3-methylcholanthrene (MC) induced AHH. One class of flavones, typified by 7,8-benzoflavone, activates constitutive AHH and inhibits the MC-induced AHH. A second class, typified by maackiain, exhibits effects which are the reverse of 7,8-benzoflavone. The flavones we screened may prove useful in studies which characterize the cytochrome P-450 content of different tissues and should be useful in exploring the mechanism of action of these isozymes.

PHS 6040 (Rev. 1/84)

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

H.V. Gelboin	Chief	LMC NCI
F. K. Friedman	Senior Staff Fellow	LMC NCI

Objectives:

To investigate naturally occurring inhibitors which are present in our environment and to characterize their effects on cytochromes P-450 from animal and human tissues.

Methods Employed:

1) Naturally occurring flavonoid compounds, some of which are purified from plants and some of which are chemically synthesized, are used; 2) microsomal preparations are made from liver of nontreated, phenobarbital (PB)- and MC-treated rats; and 3) aryl hydrocarbon hydroxylase activity is determined for these preparations in the absence and presence of inhibitors.

Major Findings:

7,8-Benzoflavone, maackiain, medicarpin, and related compounds are inhibitors that affect different forms of AHH. 7,8-Benzoflavone inhibits the AHH from MC-treated rat tissues, but has little or no effect on the AHH of untreated or PB-treated rat tissues. Maackiain and medicarpin greatly inhibit the AHH from noninduced and PB-induced rat tissues, but have little effect on the AHH from MC-treated rat tissues.

Significance to Biomedical Research and the Program of the Institute:

Maackiain, 7,8-benzoflavone, and the other flavones tested are useful modulators of specific forms of AHH in human as well as animal tissues. The different specificities of these compounds renders them valuable tools for probing the structure and function of cytochrome P-450, assessing the factors that influence polyaromatic hydrocarbon metabolism, and clarifying the relationship between drug and carcinogen activation and detoxification. Some of the compounds under study may be powerful natural inhibitors or modulators of chemical carcinogen-induced cancer and may be present in the human diet.

Proposed Course:

To examine the effects of related flavonoids. To investigate the interaction of these flavones and related compounds with various forms of cytochrome P-450.

Publications:

Friedman, F. K., West, D., Dewick, P. M. and Gelboin, H. V.: Specificity of medicarpin and related flavones in inhibition of rat hepatic mixed function oxidase activity. Pharmacology (In Press)

Friedman, F. K., West, D., Sugimura, T. and Gelboin, H. V.: Flavone modulators of rat hepatic aryl hydrocarbon hydroxylase. Pharmacology (In Press)

Friedman, F. K., Wiebel, F. J. and Gelboin, H. V.: Modulation of rat hepatic aryl hydrocarbon hydroxylase by flavones and polycyclic aromatic hydrocarbons. Pharmacology (In Press)

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z10CP05208-05 LMC

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Phenotyping of Cytochrome P-450 in Human Tissues Using Monoclonal Antibodies

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Haim Kapitulnik	Expert	LMC	NCI
Others:	S.S. Park	Senior Staff Fellow	LMC	NCI
	H.V. Gelboin	Chief	LMC	NCI

COOPERATING UNITS *(if any)*

Roswell Park Memorial Inst., Buffalo, NY (H. Gurtoo)
University of Colorado Medical School, Denver, CO (P. Manchester, K. Gottlieb)

LAB/BRANCH

Laboratory of Molecular Carcinogenesis

SECTION

Metabolic Control Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.0	1.0	0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects X ☐ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The diversity of the cytochromes P-450 in human tissues, i.e., liver and placenta was investigated using monoclonal antibodies (MAbs) to 3-methylcholanthrene (MC) and phenobarbital (PB)-induced rat liver cytochrome P-450. The content of epitope-specific cytochrome P-450 was determined by competitive radioimmunoassay (RIA) using a panel of MAbs. The isozymic content varied among different individuals and tissues. The content of antigenically unique types of cytochrome P-450 can therefore be measured in different individuals and thus provide important information on their capacity for drug activation to toxic metabolites and their susceptibility to chemical carcinogens.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

Haim Kapitulnik	Expert	LMC	NCI
Sang S. Park	Senior Staff Fellow	LMC	NCI
Harry V. Gelboin	Chief	LMC	NCI

Objectives:

Differences in the profile of cytochromes P-450 present in different tissues may account for differences in metabolism that result in either the carcinogenic activation or the detoxification of drugs, mutagens, and potential carcinogens. We are using cytochrome P-450 specific monoclonal antibodies (MAbs) to investigate the diversity and multiplicity of these enzymes by 1) development of a phenotypic description of the number and quantity of cytochromes P-450 in tissues and individuals; 2) determination of their role in the detoxification or activation of specific xenobiotics; 3) determination of their role in individual variation in drug and carcinogen responsiveness; and 4) examination of the complex interactions and influence of hereditary and environmental factors.

Methods Employed:

Monoclonal antibodies were obtained from hybridomas made by the fusion of myeloma cells and spleen cells derived from BALB/c mice that had been immunized with MC- or PB-induced rat liver cytochrome P-450. Human adult liver microsomes were prepared from fresh samples of human liver obtained from kidney transplant donors. Human placental microsomes were prepared from placentas of women who are smokers or nonsmokers. A competitive radioimmunoassay (RIA) for cytochrome P-450, previously developed in this laboratory, which uses MAbs to MC- and PB-induced rat liver cytochrome P-450, was adapted for the determination of cytochrome P-450 isozymes in adult human liver and placenta. Human liver microsomes were used instead of rat liver microsomes as the standard competitor bound to the solid phase of the RIA.

Major Findings:

The analysis of competitive binding data of five ^{3}H-labeled MAbs demonstrated the presence of multiple epitope-specific cytochromes P-450 in each of five adult human liver microsome preparations. The RIA detected a three to four-fold interindividual variability in cytochrome P-450 epitope content among the five human livers. Placental microsomes from smokers and non-smokers competed with the human liver microsomes for the binding of MAbs, suggesting epitope similarities between cytochromes P-450 in these human tissues.

Significance to Biochemical Research and Program of the Institute:

Monoclonal antibodies provide us with the ability to analyze the complicated phenomenon of polycyclic hydrocarbon metabolism from a new perspective. Use of monoclonal antibodies to cytochrome P-450 should identify those P-450 species

involved in carcinogen activation and clarify the metabolic pathways responsible for chemical carcinogenesis. Knowing the enzymatic steps leading to the activation of the carcinogen will be useful for the detection and prevention of chemical carcinogenesis.

Proposed Course:

Additional MAbs will be explored as a tool for the study of human cytochromes P-450. These might recognize the different epitopes of the multiple forms of cytochrome P-450. Mabs will help define catalytic specificities of the various cytochromes P-450 by studying their inhibitory effect on metabolism of multiple substrates. MAbs may be utilized to phenotype cytochromes P-450 in tissues, organs and individuals. Such information is useful in determining the relationship between ctyochrome P-450, drug activation, and susceptibility to chemical carcinogens.

Publications:

Fujino, T., Gottlieb, K., Manchester, D. K., Park, S. S., West, D., Gurtoo, H. L., Gelboin, H. V.: Monoclonal antibody phenotyping of inter-individual differences in cytochrome P-450 dependent reactions of single and twin human placenta. Cancer Res. 44: 3916-3923, 1984.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01CP05317-03 LMC

PERIOD COVERED
October 1, 1984 to September 30, 1985

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)
Opal Suppressor tRNA Genes in Human, Chicken, Rabbit and Xenopus Genomes

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	Dolph Hatfield	Research Biologist	LMC	NCI
Others:	Karen Pratt	Guest Researcher	LMC	NCI
	Jossi Deutsch	Expert	LMC	NCI

COOPERATING UNITS (if any)
None

LAB/BRANCH
Laboratory of Molecular Carcinogenesis

SECTION
Protein Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2	2	0

CHECK APPROPRIATE BOX(ES)
☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

An evolutionary study of the sequence of opal suppressor tRNA genes and their flanking sequences (i.e., in human, rabbit, chicken and Xenopus genomes) has been completed. Previously, a chicken and a human opal suppressor tRNA gene were isolated and sequenced and this past year the sequences of the corresponding genes, which had been isolated from rabbit and Xenopus genomes, were determined. The human and rabbit genes are strictly conserved as are those of chicken and Xenopus; and the mammalian genes differ from the others by a single pyrimidine transition at position 11. The gene, therefore, has been highly conserved which provides strong evidence that the gene products have an important cellular function. In addition, two gene products which are known to form phosphoseryl-tRNA arise from the single opal suppressor tRNA gene. The fact that these tRNAs are phosphorylated on the serine moiety and read the nonsense codon UGA suggest that they have a specialized and unique function which lies outside our present concepts of tRNA utilization. The tRNA gene products are present in minor levels intracellularly compared to other seryl-tRNA isoacceptors. Both the 5' flanking region, which has been highly conserved between rabbit and human genomes and has short stretches of homology in all four species, and the 5' internal control region, which has two extra nucleotides compared to the corresponding segment in all known tRNA genes, may have a regulatory role in reducing the level of transcription of the opal suppressor tRNA genes. These possibilities are being investigated by replacing the 5' flanking region with that of another tRNA gene and by site-specific mutagenesis of the 5' internal control region. Oligonucleotides have been synthesized which have a single or double base change in the 5' internal control region and in the anticodon and are being used in mutagenesis studies to convert the 5' internal control region to a normal tRNA control region and to convert the opal suppressor tRNA gene to an ochre suppressor.

PHS 6040 (Rev. 1/84)

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

Dolph Hatfield	Research Biologist	LMC	NCI
Karen Pratt	Guest Researcher	LMC	NCI
Jossi Deutsch	Expert	LMC	NCI

Objectives:

The major goal of the project is to understand the structure and function of opal suppressor tRNA genes in higher vertebrates, and the role of these genes in protein phosphorylation.

Specific steps to achieve this goal are:

1) Isolate and characterize opal suppressor tRNA genes from chicken, human, rabbit, and Xenopus DNA libraries; 2) sequence the genes and their flanking DNA segments; 3) investigate the structure of the genomic regions that contain these genes with respect to transcription and evolutionary conservation; 4) study the control of transcription using in vitro and in vivo transcription systems; 5) use in vivo transcription systems to study processing and localization of the tRNA product; 6) make site-specific mutations in the promoter and anticodon regions of tRNA genes and to replace the 5' flanking sequence with that of another tRNA gene in order to understand better their expression and cellular function; and 7) subclone the opal suppressor tRNA gene which has the 5' flanking sequence replaced so that it will make product in high yields and subclone the ochre suppressor tRNA gene which was generated by site-specific mutagenesis into a mammalian cell line in order to determine the effects of these suppressors on cellular function (i.e., if the gene products are phosphorylated on the serine moiety and if phosphoserine is incorporated directly into protein).

Methods Employed:

The purified opal suppressor tRNA and the opal suppressor tRNA genes are prepared as probes, genes are identified in restriction digests, gene fragments are purified, isolated and subcloned, restriction maps derived, and transcription determined.

Human and chicken opal suppressor tRNA genes have been subcloned into the single stranded DNA bacteriophage M13. The chicken gene is cloned into M13 mp19 and the human gene is cloned into M13 mp18 and um20. The genes are oriented into M13 such that the coding strand of each gene is carried with the phage plus strand. Oligonucleotides which are identical to a sequence in the anticoding strand of the gene with the exception of a one or two base difference are synthesized by chemical techniques. The oligonucleotides are then hybridized to the (single stranded) complementary region of the gene subcloned into M13. The oligonucleotide serves as a primer and the remainder of the gene and vector

are resynthesized with Klenow fragment. Following ligation of the newly synthesized strand, host cells are infected with the replicative form. Plaque lifts are made and screened with ^{32}P-oligonucleotide by increasing the temperature of the wash steps in removing the probe. Phage carrying the mutated DNA are detected by their ability to retain the ^{32}P-oligonucleotide at elevated temperatures.

MAJOR FINDINGS:

Four opal suppressor tRNA genes and two pseudogenes have been sequenced from higher vertebrates. The four normal genes code for the opal suppressor tRNAs. They are identical in sequence in the human and rabbit genomes and differ by only a single U to C change at position 11 from the corresponding genes in chicken and Xenopus. The fact that the opal suppressor tRNA gene has not undergone evolutionary change in these organisms provides strong evidence that the gene products play an important role in cellular function.

The human and rabbit genomes contain a second gene which has been determined to be a pseudogene. The human pseudogene was previously shown to be truncated near the 3' terminus such that the 5' and 3' ends cannot form a tRNA cloverleaf. The 3' half of the rabbit pseudogene has been truncated at the base which is immediately 3' to the anticodon. The avian genome does not contain a pseudogene and a pseudogene was not found in Xenopus. The significance of the opal suppressor tRNA pseudogene in mammalian genomes is not clear.

The 5' flanking sequences of the human and rabbit genomes show extensive homology. This homology is not present in the flanking sequences of the pseudogenes of these organisms. Furthermore, short stretches of common sequences occur in the 5' flanking regions of each of the four organisms. These observations strongly suggest that the 5' flanking regions have a regulatory function in the expression of the opal suppressor tRNA genes.

All four genes possess a TATA box in their 5' flanking region. TATA boxes are associated with transcription of mRNA genes by RNA polymerase II. There is no evidence that the opal suppressor tRNA genes are transcribed by Pol II. In fact, transcription studies with extracts of Xenopus oocyte nuclei demonstrate that the avian and human genes are transcribed by the normal tRNA polymerase, Pol III. Therefore, the significance of the TATA boxes is not understood at present.

Two minor seryl-tRNAs are known to occur in the seryl-tRNA population of human reticulocytes and chicken, Xenopus and rabbit livers. Presently, only a single normal gene has been found in the genomes of these organisms. Therefore, it appears that a single opal suppressor gene encodes two tRNA products. The most interesting observation in this finding is that a base in the anticodon and four other bases in the 5' 40% of the tRNAs must undergo pyrimidine transitions post-transcriptionally.

The oligonucleotide which corresponds to the anticodon with the exception of a single base change has been used to convert the opal to an ochre suppressor. The ochre gene will be sequenced to confirm the single base change. We plan to subclone the ochre suppressor into an appropiate vector and introduce it into

mammalian cells to determine the effects of an ochre suppressor which may be capable of forming phosphoseryl-tRNA on cellular function. The oligonucleotide which lacks two nucleotides in the 5' internal control region of the gene has failed to serve as a primer for resynthesis of the gene and vector. Therefore, two additional oligonucleotides are being synthesized which would remove the extra nucleotides in the 5' internal control region one base at a time.

Significance to Biomedical Research and the Program of the Institute:

The function of suppressor tRNAs which have phosphoserine attached to them is very unusual and apparently is unique in higher vertebrates. It is important to understand this special means of protein phosphorylation in light of new information relating protein phosphorylation directly to carcinogenesis.

Proposed Course:

1) Determine where transcription of these tRNAs initiates, how processing proceeds, and where the final tRNA product is localized in the cell (nucleus vs. cytoplasm); 2) complete the studies on site-specific mutations in the promoter and anticodon regions of opal suppressor tRNA genes and replace the 5' flanking region of the gene with a corresponding region from a tRNA gene which is very active in transcription in order to better understand the expression and function of these genes; and 3) subclone the opal and ochre suppressor tRNA genes into an appropiate vector for introducing them into mammalian cells.

Publications:

Green, M., Hatfield, D. Miller, M. and Peacock, A.: Prolactin homogeneously induces the tRNA population of mouse mammary explants. Biochem. Biophys. Res. Comm. 129: 233-239, 1985.

Hatfield, D.: Suppression of termination codons in higher eukaryotes. Trends Biochem. Sci. 201: 201-204, 1985.

O'Neill, V., Eden, F., Pratt, K. and Hatfield, D.: A human opal suppressor tRNA gene and pseudogene. J. Biol. Chem. 260: 2501-2508, 1985.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05318-03 LMC

PERIOD COVERED
October 1, 1984 to September 30, 1985

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Immunopurification and Characterization of Cytochrome P-450

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Fred K. Friedman	Senior Staff Fellow	LMC	NCI
Others:	Richard C. Robinson	Biologist	LMC	NCI
	Sang S. Park	Senior Staff Fellow	LMC	NCI
	Harry V. Gelboin	Chief	LMC	NCI

COOPERATING UNITS *(if any)*

None

LAB/BRANCH

Laboratory of Molecular Carcinogenesis

SECTION

Metabolic Control Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.8	0.8	1.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The cytochromes P-450 metabolize a variety of drugs and carcinogens. The multiple forms of this enzyme display unique yet broad, substrate and reaction specificity. The focus of this project is the identification, characterization, and elucidation of structure-function relationships of these isozymes. Monoclonal antibodies (MAbs) to specific cytochromes P-450 are an essential tool in these studies. Several cytochromes P-450 have been substantially purified in a one-step immunoadsorption procedure using Sepharose bound MAbs to the major forms of rat liver cytochrome P-450 induced by 3-methylcholanthrene and phenobarbital (MC-P-450 and PB-P-450, respectively). When mixed with solubilized tissue microsomes, the immunoadsorbents bind polypeptides which are readily desorbed at pH 3.0. Cytochromes P-450 have been purified with MAbs 1-7-1 and 1-31-2 from livers of C57Bl/6 and DBA/2 mice, guinea pigs and hamsters, and from rat lung. Such immunoadsorption experiments based on different MAbs reveal epitope relatedness between cytochromes P-450 in different tissues, strains, and species. The cytochromes P-450 isolated by this procedure were analyzed structurally by peptide mapping and NH_2-terminal amino acid analysis. Varying degrees of homology of these cytochromes P-450 were found. The hepatic cytochromes P-450 isolated from MC- and PB-induced rats exhibit small yet detectable levels of enzyme activity. A higher level of active cytochrome P-450 was prepared by a novel antigen-exchange method in which inactive denatured P-450 was exchanged for native cytochrome P-450 bound to the immunoadsorbents.

PHS 6040 (Rev. 1/84)

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

Fred K. Friedman	Senior Staff Fellow	LMC	NCI
Richard Robinson	Biologist	LMC	NCI
Sang S. Park	Senior Staff Fellow	LMC	NCI
Harry V. Gelboin	Chief	LMC	NCI

Objectives:

To identify, purify, and characterize the multiple forms of cytochrome P-450 in animal and human tissues. Monoclonal antibodies (MAbs) are utilized as highly specific reagents for recognition of individual cytochromes P-450 on the basis of epitope content.

Methods Employed:

MAbs were prepared to several liver microsomal cytochromes P-450 from rats treated with inducing agents such as 3-methylcholanthrene (MC) and phenobarbital (PB). The MAbs were covalently linked to Sepharose to yield an immunoadsorbent. This resin was combined with microsomes from various tissues and species, and the proteins bound to the column characterized upon elution from the resin. Analytical methods employed include gel electrophoresis, spectral analyses, and measurements of aryl hydrocarbon hydroxylase (AHH) and 7-ethoxycoumarin deethylase (ECD) activities.

Major Findings:

Spectral and SDS-gel analyses demonstrate that a fraction of the total cytochrome P-450 in microsomes from MC-and PB-treated rats binds tightly to the immuno-adsorbents used. Acid conditions (pH 3) are necessary to elute bound cytochrome P-450. Using Sepharose immunoadsorbents based on the MAbs 1-7-1 and 1-31-2, both to MC-induced P-450, cytochrome P-450 was purified from MC-induced livers of rats, C57Bl/6 and DBA/2 mice, hamsters, and guinea pigs.

The purified hepatic cytochromes P-450 were analyzed for structural homology by peptide mapping studies with SDS-gels and HPLC. The peptide patterns revealed varying degrees of structural homology among these cytochromes P-450. The purified cytochromes P-450 were subjected to NH_2-terminal amino acid sequence analysis. The sequences of the rat and mouse isozymes were consistent with those in the literature. The guinea pig isozyme sequence was determined for the first time and exhibited limited homology with the other isolated isozymes.

An MC-induced cytochrome P-450 was immunopurified from rat lung. It appeared similar to the major MC-induced hepatic isozyme on the basis of apparent molecular weight, peptide map on an SDS-gel, and NH_2-terminal sequence. We have modified our immunopurification procedure, which denatures the cytochrome P-450 during the desorption step, to obtain an enzymatically active species. When inactive enzyme was added to a Sepharose-MAb which had

absorbed active cytochrome, the latter was displaced from the immunoadsorbent as measured by AHH and ECD activities.

We have immunopurified from the human AHH-1 lymphoblastoid cell line a 45 kD protein which specifically binds several of our MAbs. This polypeptide may represent a human cytochrome P-450.

Significance to Biomedical Research and the Program of the Institute:

Purification of individual cytochrome P-450 by a simplified procedure using specific MAbs offers a new approach to studying their multiplicity. Cytochrome P-450 isozymes obtained in the manner from different tissues and species can be further characterized. The methodologies developed can then be applied to human tissues. The relationship of type and amount of cytochrome P-450 to drug and carcinogen metabolism can then be assessed. The antigen-exchange technique should be generally applicable to preparation of native proteins by immunoadsorption methods.

Proposed Course:

The individual forms of cytochromes P-450 will be subjected to various physical-chemical structural analyses using MAbs as specific probes of function and structure. Efforts to obtain catalytically active cytochrome P-450 will continue since characterization of activities is most relevant to our goal of relating isozyme phenotype to metabolic activity. Such detailed characterization should aid in gaining further insight into the role of cytochrome P-450 multiplicity in the metabolism of various drugs and carcinogens. Some emphasis will be placed on isolating isozymes from human tissues.

Publications:

Cheng, K. C., Gelboin, H. V., Song, B. J., Park, S. S., and Friedman, F. K.: Detection and purification of cytochromes P-450 in animal tissues with monoclonal antibodies. J. Biol. Chem. 259: 12279-12284, 1984.

Cheng, K. C., Krutzsch, H. C., Park, S. S., Grantham, P. H., Gelboin, H. V. and Friedman, F. K.: Amino-terminal sequence analysis of six cytochrome P-450 isozymes purified by monoclonal antibody directed immunopurification. Biochem. Biophys. Res. Commun. 123: 1201-1208, 1984.

Friedman, F. K., Park, S. S., Fujino, T., Song, B. J., Cheng, K. C. and Gelboin, H. V.: Phenotyping cytochromes P-450 with monoclonal antibodies. In Cooke, M. and Dennis, A. (Eds.): Proceedings of the Ninth International Symposium on Polynuclear Aromatic Hydrocarbons. Columbus, Battelle. (In Press)

Friedman, F. K., Park, S. S. and Gelboin, H. V.: The application of monoclonal antibodies for studies of cytochrome P-450. Rev. Drug Metab. Drug Interact. (In Press)

Friedman, F. K., Pastewka, J. V., Robinson, R. C., Park, S. S., Marletta, M. A. and Gelboin, H. V.: Monoclonal antibodies to cytochrome P-450 immuopurify a 45 kD protein from a human lymphoblastoid cell line. FEBS Lett. (In press).

Friedman, F. K., Robinson, R. C., Krutzsch, H. C., Grantham, P. H., Park, S. S. and Gelboin, H. V.: Monoclonal antibody directed isolation and amino-terminal sequence analysis of phenobarbital induced rat liver cytochromes P-450. (In Press)

Friedman, F. K., Robinson, R. C., Park, S. S. and Gelboin, H. V.: Catalytic activity of cytochromes P-450 purified by monoclonal antibody-directed immunopurification. Biochem. Pharmacol. 34: 2051-2054, 1985.

Friedman, F. K., Robinson, R. C., Song, B. J., Park, S. S., Crespi, C. L., Marletta, M. A. and Gelboin, H. V.: Monoclonal antibody directed determination of cytochrome P-450 types expressed in a human lymphoblastoid cell line. Mol. Pharmacol. (In Press)

Gelboin, H. V. and Friedman, F. K.: Monoclonal antibodies for studies on xenobiotic and endobiotic metabolism: cytochromes P-450 as paradigm. Biochem. Pharmacol. (In Press)

Gelboin, H. V., Park, S. S., Fujino, T., Song, B. J., Cheng, K. C., Miller, H., Robinson, R., West, D. and Friedman, F. K.: Cytochrome P-450, xenobiotic and endobiotic metabolism: Monoclonal antibody directed detection, purification and reaction phenotyping. In Boobis, A. R., Caldwell, J., deMatteis, F. and Elcombe, C. R. (Eds.): Microsomes and Drug Oxidations. London, Taylor and Francis, 1985, pp. 390-401.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05339-03 LMC

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Radioimmunoassay of Cytochromes P-450 Using Monoclonal Antibodies

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	B. J. Song	Visiting Fellow	LMC	NCI
Others:	S. S. Park	Senior Staff Fellow	LMC	NCI
	F. K. Friedman	Senior Staff Fellow	LMC	NCI
	H. V. Gelboin	Chief	LMC	NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Molecular Carcinogenesis

SECTION

Metabolic Control Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.1	1.1	0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The multiplicity of cytochromes P-450 is being studied with monoclonal antibodies (MAbs) to 3-methylcholanthrene (MC)-induced rat liver cytochrome P-450. A semiquantitative, direct radioimmunoassay (RIA) has been developed to measure cytochrome P-450 in the microsomes from various tissues in animals that are untreated, or treated with MC. The amounts of cytochrome P-450 in different tissues and species, including human samples such as placentas and lymphocytes, were examined by competitive RIA. Individual differences have been observed by this method, which is more reliable than measurements of enzyme activity. Placentas from women who smoked cigarettes contained greater amounts of cytochrome P-450 with the MAb-specific epitope than placentas from nonsmokers. The amount of MAb-specific cytochrome P-450 in human peripheral lymphocytes increased after treatment with benz(a)anthracene. RIAs with multiple MAbs have also been used to detect epitope-specific cytochromes P-450 in animal livers, with the goal of classifying various tissues with respect to MAb-specific cytochromes P-450. Much higher levels of cytochrome P-450 recognized by MAb 1-7-1 were observed in MC-treated rats and C57BL/6 mice than in untreated rats, MC-treated DBA/2 mice and guinea pigs. These analyses provide an approach to the study of cytochrome P-450 multiplicity that is complementary to enzymatic and structural studies. RIA methods will aid in defining the epitope-specific cytochromes P-450 content in different tissues, species, and strains of laboratory animals, and in understanding the diversity of the cytochromes P-450 and their role in individual susceptibility to carcinogenesis.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

B. J. Song	Visiting Fellow	LMC	NCI
S. S. Park	Senior Staff Fellow	LMC	NCI
F. K. Friedman	Senior Staff Fellow	LMC	NCI
H. V. Gelboin	Chief	LMC	NCI

Objectives:

In order to understand the detailed genetics and role of cytochromes P-450 in carcinogen and drug metabolism, a phenotypic description of the type and amount of isozymes in various species and tissues is required. Monoclonal antibodies (MAbs) to 3-methylcholanthrene (MC)-induced rat liver cytochrome P-450 are used as highly specific probes in the development of radioimmunoassays (RIAs) for different cytochromes P-450.

Methods Employed:

Monoclonal antibodies to rat liver cytochromes P-450 were prepared by general methods. The microsomes were prepared from different tissues from species which were untreated, or treated with MC. Rat, hamster, guinea pig, and the mouse strains, C57BL/6 and DBA/2, were studied along with human placentas and lymphocytes. The cytochrome P-450 content of the microsomes from these tissues were examined by either competitive RIA or a direct RIA using a rat MAb against mouse IgG. The MAbs used in this study were labeled by [^{35}S] or [^{3}H]. The RIA data were compared with enzymatic activities such as aryl hydrocarbon hydroxylase (AHH) in the absence and presence of MAb.

Major Findings:

Monoclonal antibodies are a useful tool in the identification of specific cytochromes P-450 in crude microsome preparations, as evidenced by solid-phase RIA. In rat liver, competitive RIA indicated that there is considerable elevation in the level of the form of cytochrome P-450 specific for MAb 1-7-1 in MC-treated rats, relative to the levels in control rats. Differences in MAb-specific cytochrome P-450 content were also observed among livers from C57BL/6 mice, hamster, guinea pig, and DBA/2 mice. The competitive RIA data were confirmed by direct RIA analysis with a double MAb technique. The RIA-detectable, species-dependent differences in the amount of MC-induced cytochrome P-450 is consistent with the corresponding data on AHH activity and immunopurification.

The human placentas and lymphocytes exhibited a MAb 1-7-1 specific epitope in common with rat liver MC-induced microsomes. The cytochromes P-450 responsible for the AHH induced by smoking was observed in human placenta microsomes, whereas little was detected in placentas from nonsmokers. The antigen appeared to be more stable than AHH activity; at 21°C and 4°C, as well as during pH changes; thus the RIA is a more reliable and sensitive analytical tool in phenotyping cytochrome

P-450 than enzyme activity measurements. RIA has been used to define the epitope content of P-450s in various tissues. Competitive RIAs also have helped to define overlapping specificities of MAbs. In addition we have developed a generalized RIA with rat MAb 187.1, specific for mouse MAbs. With this rat MAb we can readily screen many samples of tissues for the presence of specific P-450s recognized by our library of mouse MAbs, and thus compare the MAb-specific P-450 contents of multiple samples.

Significance to Biomedical Research and the Program of the Institute:

Development of RIAs will aid in the efficient establishment of an atlas of MAb-defined cytochromes P-450, which can be used in numerous studies relating to cytochrome P-450 multiplicity and genetic control. It will also help to determine the role of particular cytochromes P-450 in drug and carcinogen metabolism. A rapid, efficient RIA system that detects and phenotypes P-450 isozymes in human tissues will aid in assessment of individual differences in cytochrome P-450 content and susceptibility to carcinogenesis.

Proposed Course:

Our RIA procedures will be refined to develop a quantitative, sensitive, and reproducible method for detection of cytochromes P-450 that are recognized by specific MAbs. Tissue microsomes from different species will be analyzed. The RIA will also be applied to human tissues such as lymphocytes to examine inter-individual differences. RIA will also be used as a tool for structural analysis of purified cytochromes P-450 with respect to epitope content.

Publications:

Song, B. J., Gelboin, H. V., Park, S. S., Tsokos, G. amd Friedman, F. K.: Monoclonal antibody-directed radioimmunoassay detects cytochromes P-450 in human placenta and lymphocytes. Science 228: 490-492, 1985.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05436-01 LMC

PERIOD COVERED

October 1, 1984 through September 30, 1985

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

The Role of Mouse Cytochrome P1-450 and P3-450 in Carcinogenesis

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	N. Battula	Expert	LMC	NCI
Others:	J. Sagara	Visiting Fellow	LMC	NCI
	F. Gonzalez	Senior Staff Fellow	LMC	NCI
	H. V. Gelboin	Chief	LMC	NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Molecular Carcinogenesis

SECTION

Metabolic Control Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.5	1.5	

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Polycyclic aromatic hydrocarbons induce oxidative and conjugative enzymes in responsive animals and in human cells. The oxidative enzymes, i.e., cytochrome P-450s, generate active intermediates from procarcinogens and thus initiate chemical carcinogenesis. We propose to investigate this hypothesis by directly introducing molecularly cloned full length P-450 cDNAs into different cells and analyzing the extent of binding of the carcinogen to the cellular macromolecules, and the incidence of tumors by challenging with appropriate carcinogens.

For this purpose, we plan to use the infectious eukaryotic expression vector, vaccinia virus, which was previously shown to express enzymatically active foreign proteins that are transported to the appropriate subcellular site of residence. We have introduced the mouse P1-450 and P3-450 into the recombination vector and are in the process of transferring these into the vaccinia virus to generate the infectious recombinant vaccinia virus.

PHS 6040 (Rev. 1/84)

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

N. Battula	Expert	LMC	NCI
J. Sagara	Visiting Fellow	LMC	NCI
F. Gonzalez	Senior Staff Fellow	LMC	NCI
H. V. Gelboin	Chief	LMC	NCI

Objectives:

The overall goal of the project is to determine the contribution of the inducible oxidative enzymes, aryl hydrocarbon hydroxlases to chemical carcinogenesis. Specific aims are to a) test if the inducible mouse cytochromes P_1-450 and P_3-450 are expressed in recombinant vaccinia virus infected cells, b) test if expressed P-450s are enzymatically active and transported to the endoplasmic reticulum and c) test the extent of binding of the challenging hydrocarbon to cellular macromolecules and to the incidence of tumors.

Methods Employed:

The work involves the construction of infectious recombinant vaccinia virus containing the cDNAs for the mouse cytochromes P_1-450 and P_3-450. The first step in the construction series is to transpose the cDNAs to the proper site on the insertion vector that contains vaccinia virus transcriptional promoters and segments of vaccinia DNA. The resulting recombination vector is transfected into vaccinia virus infected cells, whereupon homologous recombination results in site specific insertion of the chimeric gene into the virus genome. The recombinant genomes packaged into the infectious progeny virus are selected on appropriate cells using selective media. In addition to the genetic selection various other methods, including molecular hybridization, will be employed to distinguish the recombinant virus from its parent. The expression products of the P_1-450 and P_3-450 in the recombinant virus infected cells can be detected using a variety of analytical procedures. For example, using monoclonal and polyclonal antibodies, the antigenic products will be analyzed by immunoprecipitation, radioimmunoassay and immunoblotting. The cytochrome P-450 products will be analyzed for enzymatic activity with a highly sensitive fluorescence assay using benzo(a)pyrene as substrate. The authentic size P-450 products will be analyzed by 2-dimensional gel electrophoresis.

Major Findings:

This project was started in April 1985. We have constructed the recombination vector which contains the P-450 cDNA adjacent to vaccinia viral DNA promoter and these two sequences are sandwiched with fragments of vaccinia thymidine kinase gene. Vaccinia virus assays were standardized on human TK^- cells, HeLa cells, BSc-1 cells and CV-1 cells. These different cell lines are used for selective functions of vaccinia virus. Currently we are in the process of transferring the P-450 cDNAs onto vaccinia viral DNA to generate the infectious recombinant virus.

Significance to Biomedical Research and the Program of the Institute:

Environmental chemicals contribute to the incidence of cancer. It is widely believed (but disputed) that the inducible cytochrome P-450 isozymes mediate activation of chemicals which bind to cellular macromolecules and potentiate chemical carcinogenesis. This project is designed to test this hypothesis more directly by expressing cytochrome P-450 in different cells and test the relationships of covalent binding of carcinogens, (to macromolecules) mutagenicity and tumor incidence.

Proposed Course:

Every attempt will be made to accomplish the goals of this project using the approaches described in this report. If and when other cytochrome P-450 genes are available, these experiments will be extended to express their products and to assess their contribution to the process of chemical carcinogenesis. There are some practical problems using the recombinant infectious vaccinia virus. To overcome this, we plan to use other vectors to test the validity of this hypothesis.

Publications:

None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05437-01 LMC

PERIOD COVERED

October 1, 1984 to September 30, 1985

TITLE OF PROJECT (*80 characters or less. Title must fit on one line between the borders.*)

Transcription Regulatory Elements in the Mouse Cytochrome P-450 Gene

PRINCIPAL INVESTIGATOR (*List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Frank J. Gonzalez	Senior Staff Fellow	LMC	NCI
Others:	Daniel W. Nebert	Chief	LDP	NICHD
	Lisa Ann Neuhold	Chemist	LDP	NICHD

COOPERATING UNITS (*if any*)

None

LAB/BRANCH

Laboratory of Molecular Carcinogenesis

SECTION

Metabolic Control Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.0	1.0	1.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK (*Use standard unreduced type. Do not exceed the space provided.*)

The mouse cytochrome P_1450 metabolizes polycyclic aromatic hydrocarbons, particularly carcinogens such as benzo(a)pyrene. These can be converted to highly reactive metabolites than can produce cellular damage and lead to cell transformation. Alternatively, other metabolites can be easily conjugated with glucuronic acid, glutathione, SO_4 etc., and eliminated. The metabolic pathways through which a potential carcinogen enters depends largely on the concentration of P_1450 and other P-450 species, epoxide hydratase, and conjugating enzymes in the cell. In order to understand the mechanisms by which P_1450 levels can be elevated by drugs and chemical carcinogens, the cytochrome P_1450 gene has been cloned. The whole gene has been sequenced including 1500 bp upstream of the genes cap site. Sequences associated with transcriptional regulation by 3-methylcholanthrene and 2,3,7,8 tetrachlorodibenzo-p-dioxane (TCDD) are being studied by use of the eukaryote expression vectors, pSV2-cat and pSV2-neo. DNA from the P_1450 gene, including the RNA polymerase II promoter region, is fused to the choloramphenical acetyltransferase gene (CAT) and transfected into mouse hepa-1 cells. These cells have an active TCDD receptor and benzo(a)pyrene hydroxylase activity is readily induced. DNA deletion analysis and mutagenesis will be used to delineate regions of DNA associated with receptor-ligand interactions and transcriptional activation.

PHS 6040 (Rev. 1/84)

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

Frank J. Gonzalez	Senior Staff Fellow	LMC	NCI
D. W. Nebert	Chief	LDP	NICHD
L. A. Neuhold	Chemist	LDP	NICHD

Objectives:

1) To define specific regions of DNA that are associated with transcriptional activation of the mouse $P_1$450 gene.

2) Localize and characterize the important regulatory and enhancer elements within and around the cytochrome $P_1$450 gene.

3) Characterize regulatory and enhancer sequences with respect to their functional promoter-distance and orientation requirements

4) Determine if these elements will regulate transcription of other genes (heterologous promoters).

5) If these sequences are rigorous in structure (e.g., 11 of 12 bases required for activity) their frequency in the mouse genome will be determined by hybridization blotting studies. In addition these sequences will be searched for in the corresponding rat and human $P_1$450 genes.

Methods Employed:

Eukaryote expression vectors, pSV2-cat and pSV2-neo, have been obtained from Dr. Bruce Howard of the NCI. The $P_1$450 promoter region (1st exon) and various amounts of upstream DNA are cloned into pSV0-cat (minus SV40 promoter) by use of standard protocols. The constructed plasmids are isolated and transfected on to mouse hepa-1 cells with the calcium phosphate glycerol-shock method. Both transient expression assays (48 hours) and stable transformation assays (co-transfection with pSV2-neo-selectable marker) are being utilized. In the former assay promoter, activity is being measured in cells that have taken up thousands of molecules of plasmid in an unintegrated state. In the stable transformation assay each cell has a few (1-10) integrated copies of the plasmid. Chloramphenicol acetyl transferase activity will be measured using ^{14}C-chloramphenicol. RNA levels and transcription activity will be analyzed by use of previously described methods.

Major Findings:

By use of stable co-transformation assays it was found that all of the requlatory DNA sequence in the $P_1$450 gene lies within 1700 b.p. upstream of the cap site. In these constructs TCDD produces a 4-fold increase in transcriptional activity. Incubation of cells with TCDD plus cycloheximide resulted in a 60-fold increase

in promoter expression. Deletion analysis in constructs that contained an exogenous enhancer element revealed the existence of an endogenous enhancer between 823 and 1642 of the cap site that is absolutely required for induction by TCDD to proceed. DNA sequences that are associated with receptor-ligand interaction and potential repressor-binding is located between 823 and 389 of the cap site. The final definitive association of the TCDD receptor with upstream DNA was found by transfecting variant hepa-1 cells. These cells, which were selected based on their resistance to benzo(a)pyrene have various receptor defects. Any of the regimens that were active in inducing wild types hepa-1 cells also induced CAT activity.

Significance to Biomedical Research and the Program of the Institute:

The carcinogen metabolizing cytochrome P-450 is regulated by administration of several carcinogens such as benzo(a)pyrene, 3-methylcholanthrene, benzo(a) anthracene and TCDD, among others. The former three essentially induce their own metabolism. These compounds can either be activated to procarcinogens or can be inactivated and eliminated. Analysis of the molecular details of the induction of this enzyme is paramount to understanding the interrelationships between carcinogen exposure and carcinogenesis. These studies are a prelude to analysis of humans for carcinogen exposure and carcinogenesis. Particularly important is the individual genetic differences in carcinogen exposure risks. These differences may be at the level of gene regulation.

Proposed Course:

1) Detailed deletion and point mutagenesis of constructs to define and separate enhancer, repressor-associating, and receptor-ligand associating DNA domains.

2) Comparison of mouse regulatory regions to the human P-450 gene.

Publications:

Gonzalez, F. J., Kimura, S. and Nebert, D. W.: Comparison of the flanking regions and introns of the mouse 2,3,7,8-tetrachlorodibenzo-p-dioxin-inducible cytochrome P_1450 and P_3P450 genes. J. Biol. Chem. 260: 5040-5049, 1985.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05438-01 LMC

PERIOD COVERED

October 1, 1984 - September 30, 1985

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Experimental Model Systems for Human Colon Cancer

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	T. Kakefuda	Medical Officer	LMC	NCI
Other:	H. Imai	Visiting Fellow	LMC	NCI
	C. C. Harris	Chief	LHC	NCI
	S. M. Plummer	Visiting Fellow	LHC	NCI

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Molecular Carcinogenesis

SECTION

Office of Chief

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.0	1.0	1.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The topic of the present study is the establishment of experimental model systems for the study of human colon cancer which may have been caused by genotoxic compounds produced from the natural diet. Fecapentaene-12 (fec-12), which has been recently reported to account for mutagenicity in human fecal extracts (Bruce et al.) is investigated extensively for its cellular and molecular interactions associated with its carcinogenic potentials. The mutagenicity of fec-12 is assayed with Salmonella TA104, TA100, and TA98. Fec-12 is highly mutagenic to TA100 and TA104 without metabolic activation. To investigate the molocular mechanisms of mutation, the plasmid mediated mutation assay, which was established in our laboratory, was utilized. Fec-12 is highly cytotoxic to normal human fibroblasts and is more potent in DNA repair deficient (XP) strains. Fec-12 is also mutagenic to both normal and XP cells inducing up to a 10-fold increase in 6-thioguanine resistant mutants. The interaction of fec-12 with plasmid DNA is investigated by denaturation-renaturation kinetics and electron microscopy. The damage and repair of the DNA are studied by the alkaline elution techniques with fec-12 treated human fibroblasts. These studies indicate that the genotoxicity and mutation may be mediated by cross-linking and strand breaks of the DNA induced by fec-12.

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

T. Kakefuda	Medical Officer	LMC NCI
H. Imai	Visiting Fellow	LMC NCI
C. C. Harris	Chief	LHC NCI
S. M. Plummer	Visiting Fellow	LHC NCI

Objectives:

1. To determine the molecular and cellular mechanisms of human colon cancer

2. To establish experimental model systems for the study of human colon cancer using fec-12 as a major mutagenic and carcinogenic agent.

3. To investigate the genotoxicity, mutagenicity, and carcinogenecity caused by natural diet components in the colon.

Method Employed:

Fec-12 was synthesized at the Stanford Research Institute, California. The mutagenicity of fec-12 has been tested in bacteria with Salmonella TA104, TA1000, and TA98 strains with or without the addition of microsomal S9 fractions. This laboratory has developed a highly sensitive mutation assay using plasmid pK0482 DNA which consists of amp and galactose kinase genes (galk). The plasmid DNA was modified with fec-12 prior to transfection into AB1886 (uvrA$^-$, amp^s, galk$^-$). Sequence changes and rearrangement of the DNA are studied by using pKG1820 plasmid (transcriptional terminator inserted at the upstream of the galk gene), transfected in AB1886 and then exposed to fec-12. Mutants were selected on the basis of a defunct terminator. Fec-12 interaction with DNA is investigated by incubation of EcoR1 linearized pBR322 DNA with fec-12 followed by heat denaturation and rapid quenching. An aliquot of the heat denatured and reannealed DNAs are then electrophoretically analyzed. Electron microscopic observation was carried out with the same DNA treated with fec-12. Alkaline elution of fec-12 treated cells is carried out employing standard methods established by previous studies. Human cell cytotoxicity was examined by exposing fec-12 to colon epidermal cells and fibroblasts of normal and DNA repair decicient patients. Human cell mutagenicity was determined by a change in the cells from 6-thioguanine sensitive to resistant after treatment with fec-12 using ethylnitrosourea as a positive control.

Major Findings:

The high mutagenic frequencies in bacterial strains indicates that fec-12 is a direct mutagen and that it may be enzymatically deactivated by the S9 fraction. Interaction of fec-12 with DNA is studied using a number of different laboratory techniques. The reannealing of heat denatured linear pBR322 DNA is significantly more rapid with fec-12 treated DNA than untreated DNA, a phenomenon similar to that due to trimethylpsoralen plus UV irradiation. Single and double stranded DNA treated with fec-12 demonstrates a high intensity of fluorescence, suggesting covalent binding. Electron microscopic observation of the fec-12 treated and

heat denatured DNA shows inter- and intrastrand cross-linking and an extensive fragmentation of the DNA. Alkaline elutions of fec-12 treated fibroblasts also show dose and time dependent single strand breaks and cross-linking of the DNA, supporting the findings from in vitro binding experiments. These data strongly suggest that fec-12 forms a chemically stable complex with the DNA which subsequently results in cross-linking and strand breaks. Fec-12 is highly cytotoxic to DNA repair deficient XP cells, which are significantly more sensitive to the agent than are normal human fibroblasts. Fec-12 is more mutagenic to XP cells than to normal human fibroblasts as determined by the change in 6-thioguanine resistance (study done by R. Curren and L. Yung). A recombinant plasmid DNA consisting of amp and galactose kinase genes fails to express the gene functions when the plasmid DNA is modified with fec-12 prior to transfection into the AB1886 strain of E. coli. From these results it is concluded that fec-12 causes DNA damage in human cells which results in cytotoxicity and mutation.

Significance to Biomedical Research and the Program of the Institute:

Epidemiological evidence indicates that diet may play a role in the cause of colon cancer, which is one of the most common diseases in the Western world. Substance in human feces with which the intestinal wall is in contact have been major candidates as causative agents for colon cancer. Purified fractions from feces have been shown to be strongly mutagenic in Salmonella, and fec-12 has been identified as the major mutagenic compound produced by bacterial action on feces within the colon. However, information regarding molecular interactions of fec-12 with the genetic machinery of the cell and biological consequences which follow after DNA damage is limited because of technical difficulties, the unstable and variable nature of the compound being the major problems.

Our observations that fec-12 has strong nucleophilic properties, genotoxicity, and mutagenicity have potentially important implications for the etiology of colon cancer and serve as a strong base for experimental model systems in future studies. Although the significance of fec-12 to the origin of colon cancer remains to be determined, information obtained from these studies will lead to the development of rational methods to reduce the risk of colon cancer by dietary methods or by interfering with the activity of carcinogenic agents produced in the human feces.

Proposed Course:

Further investigation on fec-12 is a logical extension toward the elucidation of the etiology of human colon cancer. Chemical interactions between fec-12 and DNA which cause cross-linking and strand breaks will be studied further using gel electrophoresis, alkaline elution and electron microscopy. Sister chromatid exchange will be studied with human fibroblasts in culture.

Antibodies specific against fec-12 modified DNA will be produced and utilized for the detection of adducts in both normal colon epidermal cells and human colon epidermal cells in culture treated with fec-12. Removal of the adducts from fec-12 treated cells by DNA repair will also be studied using these antibodies. The fec-12 DNA adducts will be isolated by HPLC and studied with NMR and other means to determine the structure of the adducts. Experiments will be carried out

in animals by producing a diverticulum on the wall of the colon with an insertion of fec-12 containing material in the pocket. These studies will provide useful information regarding the activity of fec-12 on human colon carcinogenesis.

Publication:

Slor, H., Bynum, G. D., Jr., Lee, C-H., Mizusawa, H. and Kakefuda, T.: Effect of DNA conformation on the binding of antibodies to benzo(a)pyrene diol-expoxide DNA adducts. Jpn. J. Cancer Res. (In Press).

ANNUAL REPORT OF

THE CHEMICAL AND PHYSICAL CARCINOGENESIS BRANCH

NATIONAL CANCER INSTITUTE

October 1, 1984 through September 30, 1985

The Chemical and Physical Carcinogenesis Branch (CPCB) plans, coordinates, and administers a national extramural program of basic and applied research consisting of grants and contracts, collectively concerned with the occurrence and the inhibition of cancer, caused or promoted by chemical or physical agents acting separately or together, or in combination with biological agents; plans, organizes, and conducts meetings of scientists and otherwise maintains contacts with scientists-at-large, to identify and evaluate new and emergent research in, and related to, the fields of chemical and physical carcinogenesis; provides a broad spectrum of information, advice, and consultation to scientists and to institutional science management officials, relative to the National Institutes of Health (NIH) and National Cancer Institute (NCI) funding and scientific review policies and procedures, preparation of grant applications, and choice of funding instrument, based on individual need; plans, develops, maintains, and allocates research resources necessary for the support of carcinogenesis research of high programmatic interest; and provides NCI management with recommendations concerning funding needs, priorities, and strategies relative to the support of chemical and physical carcinogenesis research, consistent with the current state of development of individual research elements and the promise of potential, new initiatives.

Research and related activities supported under this program bear upon a broad range of subject-matter areas, with principal emphasis on environmental carcinogenesis, mechanisms of action of chemical and physical carcinogens; the role of DNA damage and repair in carcinogenesis; properties of cells transformed by chemical and physical carcinogens; inter- and intra-species comparisons in the response to carcinogen exposure; the role of tumor promoters, hormones, and other cofactors in cancer causation; experimental approaches to the inhibition of carcinogenesis; the role of diet and nutrition in carcinogenesis; the role of tobacco products and smoking in carcinogenesis; and in vitro carcinogenesis studies on human and other mammalian cells, tissues, and subcellular fractions. The program also supports the synthesis, acquisition, and distribution of a considerable spectrum of chemical standards, critically needed in the field of carcinogenesis research.

Grants and contracts administered by the staff of this Branch support six complementary categories of chemical and physical carcinogenesis research and associated resources: Biological and Chemical Prevention, Carcinogenesis Mechanisms, Diet and Nutrition, Molecular Carcinogenesis, Smoking and Health, and Research Resources.

The Biological and Chemical Prevention component is concerned with the experimental inhibition of carcinogenesis caused by chemical, physical, and biological agents. Efforts are devoted to the identification, development, and testing (both in vitro and in vivo) of agents intended to inhibit carcinogenesis. Areas of prime interest include mechanisms of action of candidate preventive agents, binding proteins and receptors, structure-function relationships, and the experimental use of combinations of preventive agents.

The Carcinogenesis Mechanisms category relates to the absorption and body distribution of carcinogens; metabolism, activation, and inactivation of carcinogens; identification of proximate and ultimate carcinogenic forms; molecular structure-carcinogenicity relationships; carcinogen-mutagen relationships; isolation, identification, and synthesis of suspect carcinogens and their metabolites; factors which alter carcinogen activity; the characterization of carcinogen metabolizing enzymes; and the role of hormones in carcinogenesis.

The Diet and Nutriton category supports basic studies on the carcinogenic and anticarcinogenic effects of diet and specific nutrients in animal systems and human cells in vitro.

The Molecular Carcinogenesis component focuses on changes in biological macromolecules and in cell functions as a result of carcinogen exposure; DNA damage and repair following exposure to carcinogens; identification of biochemical and molecular markers and properties of cells transformed by carcinogens; the development of analytical procedures for the identification and quantitation of carcinogens present in biological specimens; interspecies comparisons in carcinogenesis; the role of tumor promoters and the mechanism of tumor promotion in carcinogenesis; and studies on the genetics and mechanism of cell transformation and of the genetics and regulation of enzymes characteristically associated with the carcinogenesis process.

The Smoking and Health category supports studies on the toxicology and pharmacology of smoking and tobacco-related exposures. Both grant and contract mechanisms are used to support these activities.

The Research Resources component, supported solely by contract, is principally concerned with the synthesis and distribution of selected chemical carcinogens and certain of their metabolites, with particular reference to polynuclear hydrocarbon carcinogens, their metabolic intermediates, and analogous heterosubstituted compounds, as well as the synthesis and distribution of retinoids including radiolabeled forms.

The Branch issued three RFAs this year which were the result of recommendations from workshops held previously. One was an RFA (84-CA-25) assigned to the Biological and Chemical Prevention section entitled "Innovative Approaches to Chemoprevention." The other two solicitations sought studies on the relevance of "Mutagens in Human Foods" through the use of two different funding mechanisms: the usual R01 research grant (RFA-84-CA-26) and the less familiar cooperative agreement (RFA 85-CA-01). Responses to these solicitations are still in the review and award process at this writing.

There were two contract recompetitions which resulted in RFPs this year. The first RFP was a recompetition of the "Chemical Carcinogen Reference Standard Repository" which is currently located at the IIT Research Institute in Chicago, Illinois (N01-CP-05612). The other RFP seeks to recompete an initiative known as, "Synthesis of Derivatives of Polynuclear Aromatic Hydrocarbons" which is currently held by encumbent contractors at the American Health Foundation (N01-CP-15747) and at Eagle-Picher Industries. Inc. (Chemsyn) (N01-CP-05613). Awards from these RFPs will be made in late FY 1985 and early FY 1986, respectively.

At the present time, individual research grants and contracts are supporting efforts addressing fundamental issues in chemoprevention, such as the synthesis

and discovery of anticarcinogenic agents, their efficacy in anticarcinogenesis, and the determination of their basic mechanisms of action. Many classes of chemopreventive agents are under investigation in numerous biological systems, and of these, a significant number appear promising for further development. In this regard, experience suggests that effective exploitation of new knowledge applicable to cancer prevention often requires diverse laboratory research expertise and material resources beyond the scope of most individual grants and contracts, and in many cases, beyond the capacity of single organizations. For these reasons, a request for applications (RFA) will soon be issued for NATIONAL COLLABORATIVE CHEMOPREVENTION PROJECTS (NCCPs) which are conceived as new approaches to cancer prevention in order to: acquire basic knowledge in significant biological systems for carcinogenesis/anticarcinogenesis; derive new insights into practical means for chemoprevention of the carcinogenic process; and rapidly translate these understandings into new chemopreventive entities with known ranges of efficacy and defined pharmacologic/toxicologic properties.

The Chemical and Physical Carcinogenesis Branch is proposing to establish the NCCPs with funding provided through the cooperative agreement mechanism. The cooperative agreement is an assistance mechanism in which the Government component (NIH, NCI) making the award anticipates substantial programmatic involvement with the recipient during performance of the planned activity. Each NCCP would consist of a number of laboratory research programs representing diverse scientific disciplines and expertise, such as experimental carcingoenesis, pharmacology, toxicology, medicinal and organic chemistry, molecular and cellular biology, biochemistry, immunology and pathology. Scientists in a given Project could derive from any combination of the academic, non-profit, and for-profit communities. Scientists in an NCCP could also be drawn from a single organization possessing necessary diversity and indepth expertise to accomplish Project objectives. Each Project is envisioned to consist of a Project Director, Program Leaders in several broad scientific disciplines and an NCI Coordinator. The Project Director has the responsibility for organizing the Project, assembling the multidisciplinary group of Program Leaders, preparing the cooperative agreement application and serving as Principal Investigator. This individual provides scientific and administrative leadership and, in addition, is expected to provide a laboratory program. A high degree of interaction and focus are expected in Project efforts.

It is anticipated that the the scope of an individual NCCP might include: (1) in vivo efficacy determinations in significant biological models employed in carcinogenesis studies; (2) demonstration of feasibility of any in vitro bioassays employed, as related to in vivo carcinogenesis/anticarcinogenesis; (3a) pharmacologic investigations of absorption, distribution, metabolism, and excretion with attention to dose/response relationships, b) investigations on the range of agent activity relative to organ sites at which chemoprevention is demonstrable and carcinogens/promoters against which activity exists, c) investigations characterizing the toxicologic properties of the agent; (4) biochemical investigations on mechanisms of action; and (5) investigations on structure-activity relationships elucidating chemical/structural features for agent efficacy, toxicity, and pharmacologic properties.

Solicitations such as RFAs and RFPs frequently are the result of recommendations which emerge from Branch sponsored workshops held to assess the state-of-the-art in various fields of research. This year a DCE workshop on "Neoplasia in Fish (Occurrence and Etiology)" was held for 1-1/2 days, December 12-13, 1984, at the NIH. The charge given to the invited participants, both in their letters of

invitation and verbally during the session, was 2-fold: to present overviews of the various research areas in aquatic toxicology that were most pertinent to the NCI mission, and to provide the NCI with suggestions for the most significant funding opportunities in this field. In accordance with our desire to encourage a suitably wide variety of input, there were over 80 registrants representing 24 organizations: 7 government agencies, 8 universities (including 1 Canadian), 6 research institutions, 2 private sector industrial components, and 1 State Department of Health. The workshop was co-chaired by Drs. Edward Bresnick and Pelayo Correa as representatives of the DCE Board of Scientific Counselors.

The 1-1/2 days were divided into three roughly equal segments: the first morning session being devoted to reports on invertebrates, that afternoon being devoted to finfish, and the last morning had the general thrust of aquatic chemical pollution as a human health implication.

In the last 20 to 25 years there has been a remarkable growth of interest in the study of neoplasms of poikilothermic animals. On a worldwide basis, a comparatively small number of investigators have generated a large body of information. Studies which initially, in the 1960s, focused on the description of pathologic characteristics of numerous neoplasms and their species specificity have led today to a heightened interest in aquatic animals for bioassay testing, for detection of carcinogens in the environment, and even as comparative oncology models for human cancer.

It has become evident that "cancer epidemics," or epizootics can occur in certain fish populations. At present there are at least five areas within the U.S. that appear to present significant epidemics: (1) the Puget Sound Basin in Washington, (2) Torch Lake in Michigan, (3) the Black River in Ohio, (4) the Buffalo River and (5) the Hudson River in New York. In each case the feral finfish population presents an unusually high prevalence of distinct tumor types. Tumors have been identified in several species of finfish and shellfish at one or more of the following sites: skin, gill, mantle, oral region, pharynx, stomach, pancreas, liver, kidney, gonads, heart, thyroid gland, nervous system, soft tissues, skeleton, and lymphoreticular and hematopoietic tissues.

Some of these cancers in lower animals may be similar and others dissimilar to those of man. Some cancers, in fact, arise from cells and organs of lower animals that man does not possess. There are large gaps in our knowledge about how neoplasms in aquatic animals conform to what is known about neoplasms of mammals, their morphologic characteristics, biologic course, relation to host-regulating mechanism, and their transplantability and transmissibility. Some neoplastic criteria used in mammalian pathology cannot yet be applied with confidence to many of the tumors or tumor-like lesions of aquatic species. Nevertheless, evolution of the phyla and species has imparted a great deal of biological commonage particularly at the cellular level and there are striking similarities in metabolic response to xenobiotics, at least qualitatively, between finfish and mammals. Such commonality serves as the bases for extrapolation of the significance of response at one phyletic level to that at another phyletic level.

Experimental evidence, to date, suggests that some fish species, when compared to rodents, are less sensitive to the toxic and more responsive to the carcinogenic effects of xenobiotics; they react more promptly, with a shorter latency period and with greater specificity. These characteristics, together with the fact that aquatic animals are exposed to a water environment, with all of its solubilized

and suspended components, at the level of gill, eye, gut, and skin, suggests that they should serve as major indicators of agents in the environment which pose a risk to humans. Not only are these aquatic animals obvious candidates to serve as sentinels of carcinogenic pollutants in the environment but the epizootics of cancer which they experience in confined or circumscribed water areas such as lakes and canals or sharply defined areas of rivers, bays and estuaries offer a natural experiment for establishing cause and effect relationships, interspecies comparisons, and for establishing target cells at risk.

The Branch received approval from the Board of Scientific Counselors, in May of 1985, to issue an RFA titled "Studies on the Etiology of Neoplasia in Poikilothermic Aquatic Animals: Finfish and Shellfish" which would provide up to four years of support for traditional grants (R01). Peer review will be accomplished by an ad hoc study section panel assembled by the NIH Division of Research Grants. In order to encourage applications from a diverse spectrum of scientists, particularly those with requisite expertise but presently without access to feral or laboratory aquatic animals, we propose to generate a list of scientists and laboratories that have established resources for aquatic animals and who would be receptive to discussions with individuals regarding collaboration, provision of resources, and/or consortial arrangements as appropriate. It is recognized that the expertise and logistics needed for the conduct of meaningful multidisciplinary research rarely resides in a single agency or institution and it will be a major focus of this initiative to foster new relationships which seek to encompass the required expertise. Consistent with the title of this proposed RFA are a broad spectrum of studies that would greatly facilitate our understanding of the etiology of neoplasia in finfish and shellfish. Listed below are some commonly identified needs which are intended to express the spectrum of studies of interest but which are not intended as a comprehensive list of possibilities (it will be up to the applicant to determine the scope and objective of the studies proposed):

a) Evaluation of the similarity of metabolic function in procarcinogen activation among different species of invertebrates and/or vertebrates in regard to Phase I and Phase II reactions. Assessment of the role of fish hepatocytes in metabolism of procarcinogens. Studies on bioavailability and transfer of xenobiotics and their metabolites from invertebrates to fish and from invertebrates and fish to mammals.

b) Effects of environmental and physiological variables of water temperature, age, sex and gonadal development on bioavailability and metabolism of xenobiotics.

c) Development of in vitro culture systems for normal and neoplastic cells from invertebrates and vertebrates and analysis of adducts to macromolecules of environmentally relevant xenobiotic metabolites.

d) Studies on chemical/chemical and chemical/viral interaction in the etiology of aquatic animal neoplasms and the identification of oncogenes in invertebrate and vertebrate species.

e) Analysis of DNA repair capacity, mitotic index, SCE, cell cycle time, enzyme pathways for xenobiotic metabolism under various temperature conditions in poikilothermic aquatic animals, and determination of the relationship to the persistence of genetic lesions that might lead to tumorigenesis.

f) Studies of factors involved in promotion or progression of a tumor in aquatic species. Assessment of transplantability of neoplasms.

g) The effect of chemical pollutants on the immune response in aquatic animals and the role of the immune system in aquatic animal neoplasia.

It was further proposed to, and approved by, the Board that the Chemical and Physical Carcinogenesis Branch organize a Fish Histopathology Nomenclature Working Group which would work through the logistical support of an 8(a) contractor (minority-owned small business) to develop an atlas on fish histopathology and nomenclature. At this stage in the development of this initiative, it appears that the final product would be suitable for publication possibly as a JNCI monograph.

Approximately 40 experts in tumors of particular organ systems of finfish would be identified by a steering committee of experienced fish and mammalian pathologists. It is currently envisioned that the working group would be organized into subgroups based on related organ systems. Color photographs from microslides of lesions would be classified with the many synonyms currently being used to describe them.

Funding for this endeavor, which is expected to take approximately one year, will be derived from three sources: $25,000 from the NCI, Division of Cancer Etiology; $25,000 from the Department of the Army, Medical Research and Development Command; and $25,000 from the American Petroleum Institute (API). The funds from the Army will be forwarded as an Interagency Agreement to the NCI for joint funding of the contract with Technical Resources, Inc., which has been identified as a very qualified 8(a) firm. The API funding will be negotiated directly with the contractor for support of this effort. The contractor will provide for all aspects of logistical and technical support for this effort including arrangements for travel of participants, as appropriate, providing state-of-the-art slide projection and recording capability, and the production of a draft proceedings report.

In another workshop, the current status of research on the types of adducts produced by exposure to vinyl halides, alkyl carbamates, mono and bifunctional aldehydes, epoxides, halonitrosoureas and related compounds and their role in carcinogenesis and mutagenesis was discussed at a workshop entitled "Cyclic Nucleic Acid Adducts in Carcinogenesis" which was held at the International Agency for Research on Cancer in Lyon, France on September 17-19, 1984. The workshop was primarily supported by NCI through conference grant 1 R13 CA 37358-01. Research leaders from the United States, Canada, Europe, and Japan were participants at this meeting. The Chemical and Physical Carcinogenesis Branch chose to benefit from this assembly of expertise in order to assess the current state of knowledge and determine what gaps remain in our understanding of these adducts. Dr. Bresnick, of the DCE Board of Scientific Counselors, served as a co-chairperson with Dr. Bea Singer for the final discussion session at which future research needs and approaches to understanding the biological consequences of cyclic nucleic acid adducts were discussed.

All of the chemicals that were discussed have the capacity to form exocyclic nucleic acid derivatives, many of which have been demonstrated experimentally. These included known or suspected human carcinogens (vinyl chloride, acrylonitrile, cyclophosphamide), several of which can be found in food and beverages

(ethyl carbamate, methylglyoxal, glycidaldehyde, malonaldehyde, N-nitroso-pyrrolidine), chemotherapeutic agents (haloethylnitrosoureas) and others which humans are exposed to as environmental pollutants (acrolein, also detected in cigarette smoke) or through occupational exposure (acrylonitrile, vinyl chloride). Over 40 presentations were made which focused on the occurrence, epidemiology and carcinogenic effects, chemistry and formation of exocyclic and other adducts, metabolism, biological effects, mechanistic approaches, and sensitive methods for detection of nucleic acid adducts formed from these compounds. It was clear from the presentations that a large variety of adducts could be formed with guanosine, adenosine, and cytosine. In addition, many of the compounds can also form interstrand cross-links. The mutagenicity and carcinogenicity of compounds such as vinyl chloride, acrylonitrile, methylglyoxal, ethyl carbamate and malonaldehyde were discussed. It was concluded that cyclic nucleic acid adducts could play a major role in the biological activity of these compounds, although more work is clearly needed since adducts of this type have not been identified in vivo for many compounds. The identification of adducts in DNA was determined to be a problem due to the inability to radiolabel many of the compounds to a sufficiently high specific activity. Thus, a need to develop sensitive methods for the quantitation and identification of the adducts formed was perceived. In addition, the chemical, biochemical, metabolic, and biological studies on the mutagenicity and carcinogenicity of the compounds need to be more focused and unified. This might require a multidisciplinary attack on the problem, rather than the current situation of one laboratory working on the chemistry of adduct formation with one compound but doing no biological studies and vice versa. It was also apparent that little is known about the repair of known exocyclic derivatives in mammalian cells.

The Chemical and Physical Carcinogenesis Branch received approval from the DCE Board of Scientific Counselors to issue a Program Announcement to encourage basic mechanistic studies focused on determining the formation, repair and relevance to mutagenesis and carcinogenesis of exocyclic nucleic acid derivatives. The compounds of interest which are known or are likely to form exocyclic nucleic acid derivatives include: vinyl halides (vinyl chloride, vinyl bromide), alkyl carbamates (ethyl and vinyl carbamate), halonitrosoureas (BCNU, CCNU), monofunctional unsaturated aldehydes (acrolein, crotonaldehyde), bifunctional aldehydes (glyoxal, malonaldehyde, glycidaldehyde), beta-propiolactone, acrylonitrile, N-nitroso-pyrrolidine and related cyclic nitrosamines, and some halogenated ethers and aldehydes (chloro- and bromoacetaldehyde). Examples of important areas of research emphasis include the following: 1) the identification and quantitation of adducts which may be responsible for the carcinogenicity of the test compound in animals, the transformation of cells in culture, or the mutagenicity of the compound in cells in culture or in other test systems; 2) the formation and repair of exocyclic adducts in animals, cells in culture, or other test organisms relevant to carcinogenicity, transformation and mutagenicity studies; and 3) the mechanism of mutagenesis or carcinogenesis by exocyclic nucleic acid adducts, other adducts of biological interest or cross-links which may be formed by the above mentioned compounds. It is also recognized that there will be a need to develop more sensitive methods to analyze and quantitate the many possible adducts and to detect them in DNA from cells exposed to the chosen compounds. A desired sensitive method, not widely available, is an immunoassay using monoclonal antibodies to the chosen exocyclic adduct or other relevant adduct. It is suggested that support for the development of such monoclonal antibodies may, in some cases, be appropriate for the SBIR grant/contract program as well as the traditional (R01) grant.

The CPCB also supported or contributed to the support of a number of conference grants (R13 mechanism) dealing with subjects of particular relevance to the Branch. The magnitude of this support is summarized in Table I and the titles of individual conferences can be found after each section in the Branch by looking for grants identified with an R13 prefix.

A summary of the number of grants, contracts, and associated funding relative to each of the above categories and to the Chemical and Physical Carcinogenesis Branch, as a whole, follows. Table I focuses on mechanisms of support of extramural research and related activities in the area of Chemical and Physical Carcinogenesis. Table II provides an estimate of grant and contract support, respectively, in each of the six Branch components as described above.

TABLE I
CHEMICAL AND PHYSICAL CARCINOGENESIS BRANCH
(Extramural Activities - FY 1985 - Estimated)

	No. of Contracts/Grants	$ (Millions)
Research Contracts	10	1.14
Research Grants	481	46.61
Traditional Research Grants (R01) (388 grants; $36.63)		
Conference Grants (R13) (6 Grants; $0.05 million)		
New Investigator Research Grants (R23) (14 Grants; $0.64 million)		
Program Project Grants (P01) (9 grants; $5.30 million)		
Cooperative Agreements (U01) (0 grants; $0.00 million)		
Small Business Grants (2 grants; $0.00 million)		
Outstanding Investigator Grants (2 grants; $0.82 million)		
RFAs (R01) (60 grants; $3.17 million)		
Research Resource Contracts	8	1.31
TOTAL	499	49.06

TABLE II
CHEMICAL AND PHYSICAL CARCINOGENESIS BRANCH
(Contracts and Grants Active During FY 1985)

FY 1985				
	CONTRACTS		GRANTS	
	No. of Contracts	$ (Millions)	No. of Grants	$ (Millions)
Carcinogenesis Mechanisms	0	0	99	9.57
Biological & Chemical Prevention	8	0.74	68	5.28
Molecular Carcinogenesis	0	0	260	26.44
Diet and Nutrition	0	0	42	3.31
Smoking and Health	2	0.40	12	2.01
Research Resources	8	1.31	0	0
TOTAL	18	2.45	481	46.61

SUMMARY REPORT

BIOLOGICAL AND CHEMICAL PREVENTION

The Biological and Chemical Prevention component of the Chemical and Physical Carcinogenesis Branch is responsible for research on agents that can inhibit, arrest, reverse, or delay the development of cancer in humans. Agents can derive from naturally occurring products such as foods consumed by man, from chemical synthesis, or from various biological sources. At the present time there are 68 grants in this program area with FY85 funding of approximately $5.28 million and 8 contracts with FY85 funding of approximately $0.74 million. Three additional support contracts related to this program are discussed under Research Resources.

Research grants in the program support diverse types of studies including the experimental inhibition of carcinogenesis, the inhibition or suppression of malignant transformation in culture, mechanisms of action and metabolism of preventive agents, synthesis of chemopreventive compounds, structure-function relationships, and pharmacologic disposition. Studies proceed on inhibition of carcinogenesis induced by chemical, physical and biological agents, against several stages of the tumorigenic process, and against the development of cancer at many organ sites. The modifying effects of anticarcinogens are investigated relative to a large number of biochemical and biological endpoints, which, in addition to tumorigenesis and transformation themselves, include the activity of the mixed function oxidase system, free radical generation and quenching, cell proliferation, differentiation, activation/detoxification of carcinogens, DNA repair, binding proteins or receptors for preventive agents, preneoplastic states and cytogenetic variables.

A new program initiative this year was an RFA entitled, "Innovative Approaches to Development of Cancer Chemopreventive Agents." This RFA emphasized the need for innovative approaches to expand basic knowledge and understanding of the role and mechanisms of action of chemopreventive agents in modulating the carcinogenic process. Emphasis in these objectives included pharmacokinetic investigations of promising agents for optimizing dose and delivery schedules in chemoprevention and for deriving basic understandings of absorption, distribution, metabolism and excretion, structure-activity relationships for efficacy and toxicity, animal studies of efficacy in prevention of carcinogenesis with special attention to defining the carcinogens, cocarcinogens and promoters against which activity exists, organ sites at which inhibition is possible, and the stage(s) of the carcinogenic process at which agents possess activity. Responses to this RFA are still under review.

Contracts in the program support studies on antioxidant inhibition of tumorigenesis in liver, lung, digestive tract and mammary gland; on retinoid inhibition of tumorigenesis in urinary bladder and mammary gland; on synthesis and bioassay of new retinoids for potential future development; on synthesis of large amounts of selected retinoids and studies on their toxicity, and on synthesis of radiolabeled retinoids for metabolic and pharmacologic investigations. Research accomplishments follow on a number of these endeavors.

Growth and Differentiation in Cancer Prevention: Inducers of normal or nonneoplastic differentiation are receiving increasing attention both as preventive agents in the carcinogenic process and as therapeutic agents for changing a neoplastic to a nonneoplastic phenotype. One research area very actively pursuing this approach has been the study of normal and abnormal hematopoiesis. Acute myelogenous leukemia (AML), for example, has been considered for many years as a disease reflecting blocked or arrested differentiation. Recent development of murine and human leukemic cell lines blocked at different stages of maturation now permit investigations (employing homologous populations of such neoplastic cells) centered on basic mechanisms of differentiation, on the one hand, and development of agents for prevention/therapy on the other. One such cell line employed in many investigations of myeloid differentiation is the promyelocytic leukemia cell line, HL-60. This cell line is one among many human myeloid cell lines arrested at various stages of granulocyte/macrophage differentiation. Induced differentiation of this line has been effected by a wide variety of compounds including the phorbol esters, dimethyl sulfoxide and other polar compounds, retinoic acid and other retinoids, and more recently 1 alpha, 25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$) and two fluorinated analogs of this vitamin D_3 metabolite (27). Of all of these compounds $1,25(OH)_2D_3$ is the most potent physiological inducer, effecting differentiation at nanomolar concentrations. The role of $1,25(OH)_2D_3$ as an agent responsible for mineral homeostasis has been extensively studied. Within target cells, its mechanism of action is apparently similar to that of steroid hormones in that it first binds to a specific, high affinity cytosolic receptor protein which then translocates into the nucleus, binds to chromatin and initiates genomic expression of the necessary mineral-regulating proteins.

Recently, it has been shown that the mechanism of action of $1,25(OH)_2D_3$ in the differentiation of HL-60 cells is also apparently mediated by binding to $1,25(OH)_2D_3$ receptors (27). Studies show that $1,25(OH)_2D_3$ uptake by intact HL-60 cells is specific and saturable. Extent of binding and differentiation are positively correlated. Furthermore, the $1,25(OH)_2D_3$ receptor was unequivocally characterized in HL-60 cells by sucrose density gradient centrifugation (3.3S), DNA cellulose chromatography, specific immunochemical reactivity with monoclonal antibody and selective chemical dissociation of the $1,25(OH)_2D_3$ hormone-receptor complex with the mercurial reagent p-chloro-mercuribenzene sulfonic acid. Finally, differentiation of this human-derived promyelocytic leukemia cell line possessing aggressive, malignant proliferative potential to monocyte/macrophage differentiation was determined by morphology, adherence to plastic, ability to reduce nitroblue tetrazolium, the appearance of receptor-like binding for the formyl peptide chemoattractant FMLP, the ability to phagocytose *Candida* and especially by development of the macrophage-characteristic non-specific acid esterase activity. This was important not only because previous reports had indicated granulocytic differentiation resulted from $1,25(OH)_2D_3$ treatment of HL-60 cells, but also because the potential ability of this compound to stimulate normal myeloid cells to monocyte/macrophage differentiation could comprise an important mechanism whereby $1,25(OH)_2D_3$ enhances bone resorption in vivo. This would occur because monocytes can directly resorb bone mineral and also because they themselves can apparently differentiate into bone-resorbing osteoclasts.

These results have been importantly extended by demonstrating for the first time that $1,25(OH)_2D_3$ and its fluorinated analogs do, in fact, induce macrophage differentiation, not only of human normal but also of leukemic myeloid stem cells

taken directly from patients (27). Concentrations as low as 10^{-9}M stimulate normal human marrow myeloid stem cells (GM-CFC) to preferentially differentiate to colonies containing only monocytes and macrophages, which can be compared to cultures in the absence of $1,25(OH)_2D_3$, in which normal human bone marrow GM-CFC differentiated to approximately 55% neutrophil, 10% mix and 25% macrophage colonies. Similarly, in plates containing 10^{-7}M $1,25(OH)_2D_3$ or 10^{-8}M in one of its fluorinated analogs, 80% of chronic myelogenous leukemia (CML) and approximately 50% of acute non-lymphocytic leukemia (ANLL) colony forming cells differentiated to macrophage-like cells. Though it is known that normal human monocytes contain $1,25(OH)_2D_3$ receptors and that T lymphocytes also do after activation, it is interesting to note that the paucity of GM-CFC in normal bone marrow prevents actual identification of the hormone receptor in these target cells. On the other hand, expression of $1,25(OH)_2D_3$ receptors does not itself assure that a cell can be induced to differentiate in the presence of the vitamin metabolite. KG-1 cells, for example, a human myeloblast leukemia cell line, have nearly the same number of receptors as HL-60 (which is approximately 4000 high affinity receptors/cell) yet are not triggered to undergo differentiation upon $1,25(OH)_2D_3$ exposure (27).

Antioxidants: In the last several years, the phenolic antioxidant butylated hydroxyanisole (BHA) has been shown under various conditions to both inhibit and enhance induced tumorigenesis. In addition, BHA has been reported as a carcinogen itself when fed continuously to rats at very high dietary levels for two years. Very little is actually known concerning the mechanisms involved in the various actions of this well-known compound. Recently, new information has been obtained on the efficacy and role of BHA in modulating nitrosamine carcinogenesis. Human exposure to carcinogenic nitrosamines is well known through the diet and in certain occupations (for example, dimethylnitrosamine-DMN; N-nitrosopyrollidine-NPYR; N-nitrosomorpholine-NMOR), through cigarette smoking and involuntary exposure to cigarette sidestream smoke (DMN, NPYR, other nitrosamines) and very probably from gastric nitrosation of precursor amines. The new studies investigated the effects of dietary BHA on nitrosamine induction of lung tumors in A/J mice. BHA was provided at 5mg/g of diet for one week before and through the ten weeks of exposure to three nitrosamines given separately in the drinking water. The results of this experiment, terminated 20 weeks post-carcinogen exposure, clearly demonstrate that BHA has different effects on nitrosamine lung tumorigenesis depending upon the chemical structure of the nitrosamine: strong enhancement of tumorigenicity induced by the five-membered cyclic nitrosamine NPYR; strong inhibition of tumorigenicity induced by the simple methylating nitrosamine DMN; and no effect on tumorigenicity induced by the six-membered cyclic nitrosamine NMOR. In addition, a small but statistically significant increase in lung adenomas per mouse was seen in the mice treated only with BHA for just 11 weeks compared to mice fed only the control NIH-07 diet (21).

Additional investigations have examined the effects of BHA on metabolic activation of DMN and NPYR, the two nitrosamines whose lung tumorigenicities are so strongly modulated by BHA. It was found that BHA pretreatment significantly enhances the microsomal metabolic activation of DMN in mouse lung and that methylation of lung DNA is also increased up to about four hours after a single DMN treatment. At 4 hours and out to 24 hours post-DMN treatment, 7-methylguanine levels decrease in lung DNA of BHA-fed mice relative to controls, while O^6-methylguanine levels also decrease by 8 hours, remaining less than controls out to 24 hours. A possible hypothesis for the inhibition of DMN lung tumorigenesis, in view of the metabolism and adduct studies above, is that BHA has an effect on DNA repair. In the case of

NPYR, BHA pretreatment induces both liver and lung microsomal alpha-hydroxylation. Structure-activity and metabolism studies have indicated that alpha-hydroxylation may be an important metabolic activation pathway of NPYR. Whether this is a determining mechanism for the strong enhancement by BHA of mouse lung tumorigenesis induced by NPYR remains to be seen (21).

Natural Inhibitors of Carcinogenesis: Detection and quantitation of numerous types of DNA damage are important aspects in assessing the role of cellular responses to critical injury from a variety of carcinogens and promoters. Protection of DNA from initial damage due to exogenous and/or endogenous factors, and enhancing repair activities may be important functions of some effective chemopreventive agents. Since chromosome aberration and sister chromatid exchange are cytological manifestations of DNA damage, these endpoints are frequently used. Sister chromatid exchange (SCE) is believed to reflect double-stranded DNA recombination at homologous sites of sister chromatids. Most research involving SCE analysis, however, has been limited to cell culture systems requiring medium supplementation with exogenous mitogenic agents and different types of serum, supplements known to influence the observed frequency of SCEs.

Recently, a new in vitro system has been employed for the analysis of SCE in mouse mammary epithelial cells (4). This system involves organ culture of the whole mammary gland in serum-free medium containing a mammogenic hormone mixture of insulin, prolactin, hydrocortisone and aldosterone. This organ culture system not only has certain methodological advantages (enzymatic dissociation of the tissue is not required; minimal basal levels of SCE are achieved since medium supplementation with serum or exogenous mitogenic agents is not required; exogenous microsomal activating enzymes for chemical carcinogens are not required) but also its biological significance relative to normal mammary gland development and to carcinogenesis in the mouse mammary gland have already been established (see below). The procedure is otherwise substantially similar to previous methods employing 5-bromo-2-deoxyuridine labeling of cells for differential staining of the chromatids, exposure to the test substance, colcemid incubation for metaphase arrest and chromosome preparation. Three compounds known to cause mammary gland carcinogenesis when administered to experimental animals (methylnitrosourea [MNU] dimethylbenzanthracene [DMBA] and diethylnitrosamine), tested in this system, produced significant enhancements of SCEs in the mammary epithelial cells. Further, these same carcinogens are known to cause transformation of mammary epithelial cells in this organ culture system; such transformed cells are known to give rise to mammary cancers upon transplantation to syngeneic animals. The effects of chemopreventive agents on production of SCEs in this new system has recently shown that beta-carotene, present during carcinogen exposure, drastically reduces SCEs induced by DMBA or the direct-acting carcinogen MNU. Moreover, preliminary results appear to indicate that beta-carotene is affecting neither DMBA metabolism nor DNA adduct formation in this whole mammary gland organ culture system (4).

These results on beta-carotene inhibition of DMBA-induced sister chromatid exchange importantly complement other recent work by this same group of investigators showing that beta-carotene inhibits chemical transformation of mammary epithelial cells in this whole-organ culture system (4). Treatment with beta-carotene (10^{-6}M) during DMBA exposure causes a 68% decrease in the number of glands showing nodule-like alveolar lesions (NLAL), and a reduction of 53% in the number of NLAL per transformed gland. The NLAL may be analogous to precancerous, hyperplastic alveolar nodules (HAN) in mouse mammary glands in vivo. Like HAN,

the NLAL appear to represent the product of transformed epithelial cells which have escaped from the hormonal controls of alveolar development following carcinogen treatment. They are made manifest in culture (as morphological markers of transformation) by continuing exposure after carcinogen treatment to the mitogenic, morphogenetic hormone combination of insulin, prolactin, hydrocortisone and aldosterone. Such post-carcinogen hormone treatment allows continued lobulo-alveolar growth and functional differentiation(lactogenesis), apparently providing the hormone-mediated promotional stimulus believed necessary for fixation of the transformed cells in these mammary glands obtained from immature female mice. Still further incubation in prolactin-free medium containing only insulin and aldosterone, causes complete regression (involution) of the normal alveolar structures, allowing subsequent microscopic detection of NLAL upon staining of whole mount preparations. Not only is inhibition of NLAL production found when carotene is present during carcinogen exposure, but pretreatment and post-treatment of glands relative to DMBA exposure also inhibit NLAL development. Continuous exposure to carotene results in NLAL decreases almost identical to those seen when carotene is present only during DMBA exposure. These results indicate that beta-carotene administration inhibits DMBA-induced transformation of the mammary gland in vitro at both initiation and promotion stages. Beta-carotene is a known precursor of vitamin A (retinol) and retinoids are known to inhibit or suppress transformation in culture and carcinogenesis in vivo, including DMBA- and MNU-induced mammary carcinogenesis. Although the intestine is the major site for enzymic cleavage of carotene, other organ sites such as liver, lung and skin are known to metabolize beta-carotene to retinol. In the present organ culture studies, however, accumulation of retinol was undetectable in beta-carotene treated whole mammary glands. It is possible, then, that the observed inhibition of transformation of mammary cells in vitro reflects the action of beta-carotene itself.

Inhibitors of Activated Oxygen: Activated forms of molecular oxygen may play important roles at several stages of the carcinogenic process. Specific quenching of oxygen radical production and of reactive oxygen species themselves may therefore offer an important approach to prevention of carcinogenesis. Recent studies have examined the effects of selective scavengers of superoxide ($O_2^{\cdot-}$), a proximate oxygen radical, on initiation and promotion of mouse skin papilloma formation in CD-1 mice. The biomimetic superoxide dismutase (SOD) CuDIPS (copper[II][3,5-diisopropyl-salicylate]$_2$) is one such scavenger. It is a low molecular weight, lipid soluble copper coördination complex that catalyzes the dismutation of the superoxide anion at rates comparable to native Cu/Zn SOD. In contrast to the constitutive SOD of mammalian cells which is a large metalloprotein lacking lipophilicity that doesn't penetrate well into cells when administered prophylactically, CuDIPS and other copper coordination complexes are rapidly absorbed and are believed to accumulate at intracellular sites of superoxide anion production and reactivity. CuDIPS inhibited both the initiation and promotion stages of tumorigenesis in a DMBA/TPA two-stage model system when applied topically once prior to DMBA (anti-initiation) or repeatedly prior to each phorbol ester application (anti-promotion). The promotion stage was strongly inhibited with a reduction of 60% in incidence and a decrease of 87% in papillomas per mouse. Anti-initiation effected only a modest 22% reduction in tumor yield. However, if CuDIPS was applied prior to weekly application of DMBA in a complete carcinogenic protocol, tumor incidence was decreased 25% and papillomas per mouse by 55%. Further, both incidence and tumors/mouse, produced by a single, large DMBA application, are reduced by 60% by prior CuDIPS application. The observed inhibitions of tumorigenesis are attributed to the SOD activity of CuDIPS since

the corresponding Zn (II) complex, ZnDIPS, and the ligand DIPS, both lacking SOD activity, also lacked inhibitory capacity. Further, CuDIPS pretreatment prior to either a small initiating dose of DMBA employed in two-stage carcinogenesis, or prior to a large, tumorigenic dose of DMBA, reduces covalent binding of (^{3}H)DMBA to epidermal DNA by 50-60%, suggesting that CuDIPS anti-initiating and anti-carcinogenic properties may be due to alterations in O_2-dependent metabolic activation of DMBA (26).

A series of other biomimetic superoxide dismutases has been evaluated for potential in vivo tumor-inhibitory activity using inhibition of phorbol ester-induced epidermal ornithine decarboxylase activity as an endpoint. The induction of this enzyme by tumor promoters is well correlated with their tumor promoting activities. Many inhibitors of tumor promotion inhibit the induction of ornithine decarboxylase (ODC) which catalyzes the first and rate-limiting step in polyamine biosynthesis. The dose of CuDIPS required to inhibit phorbol ester-induced epidermal ODC activity by 50% (ID50) is 1.1 micromole. The ID50 values for other SOD-mimetic copper complexes, which were synthesized, are: Cu (II) (salicylate)$_2$, 2.6; Cu (II) (3,5-ditertiarybutylsalicylate)$_2$, 2.0; Cu (II)$_2$ (indomethacin)$_4$, 0.8; Cu (II)$_3$ (hydrocortisone-21-phosphate)$_2$, 0.6; Cu (II)$_2$ (retinoate)$_4$, 0.1; Cu (II)$_2$ (acetate)$_4$, greater than 10. The most important conclusion derived from these observations is that lipophilicity of the complex appears to be necessary for biological activity, in addition to SOD activity. Finally, it was observed that while CuDIPS did not inhibit ODC induction by DMBA, it did potently inhibit the induction of ODC by other types of tumor promoters such as mezerein, teleocidin and anthralin. All of these studies indicate that reactive oxygen species may play important roles in initiation and promotion stages of carcinogenesis (26).

BIOLOGICAL AND CHEMICAL PREVENTION

GRANTS ACTIVE DURING FY85

Investigator/Institution/Grant Number	Title
1. AUERBACH, Arleen D. Rockefeller University 5 R01 CA 33948-03	Effects of Anticarcinogens on Fanconi Anemia Chromosomes
2. AWASTHI, Yogesh C. Univ. of Texas Med. Br. (Galveston) 5 R01 CA 27967-06	Mechanism of Anticarcinogenic Effect of Antioxidants
3. BAILEY, George S. Oregon State University 5 R01 CA 34732-03	Mechanisms of Inhibition of Chemical Carcinogenesis
4. BANERJEE, Mihir R. University of Nebraska (Lincoln) 5 R01 CA 25304-06	Chemical Carcinogenesis Mammary Gland Organ Culture
5. BELMAN, Sidney New York University 1 R01 CA 38156-01	Tumor Control by Onion, Garlic, and a Protease Inhibitor
6. BENEDICT, William F. Children's Hospital of Los Angeles 5 R01 CA 31574-04	Ascorbic Acid Transformation and Oncogenic Progression
7. BENSON, Ann M. Johns Hopkins University 5 R01 CA 32479-02	Modulation of Enzyme Profiles by Anticarcinogenic Agents
8. BERNSTEIN, Isadore A. University of Michigan (Ann Arbor) 5 R01 CA 32470-03	Mechanism for Retinoid Neutralization of Tumor Promotion
9. BERTRAM, John S. University of Hawaii at Manoa 7 R01 CA 39947-01	Inhibition of In Vitro Transformation by Retinoids
10. BRESNICK, Edward University of Nebraska Med. Ctr. 1 R01 CA 38150-01	Cruciferae and Carcinogenesis
11. BRINCKERHOFF, Constance E. Dartmouth College 5 R01 CA 32476-03	Action of Retinoids on Synovial Cells
12. CASSADY, John M. Purdue University West Lafayette 1 R01 CA 38151-01	Novel Natural Inhibitors of Carcinogenesis

13.	CHUNG, Fung-Lung American Health Foundation 2 R23 CA 32272-03	Screening for Inhibitors of N-Nitrosamine Carcinogenesis
14.	CROCE, Carlo M. Wistar Institute of Anatomy and Biology 5 R01 CA 32495-03	Retinoic Acid Induced Differentiation
15.	CURPHEY, Thomas J. Dartmouth College 5 R01 CA 32478-03	Pancreatic Cancer and Retinoids--Model and Mechanism
16.	DAWSON, Marcia I. SRI International 5 R01 CA 30512-04	Novel Retinoids for Chemoprevention of Epithelial Cancer
17.	DAWSON, Marcia I. SRI International 5 R01 CA 32428-03	Retinoid Tumor Inhibitory Activity-Toxicity Probe
18.	DURHAM, John P. West Virginia University 1 R01 CA 37060-01A1	Calcium/Lipid Protein Kinase in Myeloid Differentiation
19.	GOULD, Michael N. Univ. of Wisconsin (Madison) 1 R01 CA 38128-01	Anticarcinogenic Agents in Orange Peel Oil
20.	GRUBBS, Clinton J. Southern Research Institute 5 R01 CA 30986-03	Chemoprevention of Cancer Caused by Anticancer Agents
21.	HADDOX, Mari K. University of Texas Health Science Center (Houston) 5 R01 CA 32444-03	Mechanism of Retinoid Inhibition of Cell Proliferation
22.	HECHT, Stephen S. American Health Foundation 5 R01 CA 32519-03	Chemoprevention of Nitrosamine Carcinogenesis by BHA
23.	HILL, Donald L. Southern Research Institute 1 P01 CA 34968-01A1	Development of Chemopreventive Retinoids
24.	HORNSBY, Peter J. Univ. of California (San Diego) 5 R01 CA 32468-03	Antioxidant Action in a Model Cell Culture System
25.	JOHNSON, Eric F. Scripps Clinic and Res. Fdn. 5 R01 CA 34910-03	Modulation of Carcinogen Activation/Detoxification

26.	KENNEDY, Ann R. Harvard University 1 R01 CA 38246-01	Suppression of DMH-Induced Colon Cancer
27.	KENSLER, Thomas W. Johns Hopkins University 1 R01 CA 36380-02	Biomimetic Superoxide Dismutases as Antitumor Promoters
28.	KOEFFLER, H. Phillip Univ. of California (Los Angeles) 5 R01 CA 33936-03	Action of Retinoids on Myeloid Leukemia Cells
29.	KRINSKY, Norman I. Tufts University 5 R01 CA 32524-03	Anticarcinogenic Mechanisms of Carotenoid Pigments
30.	LAM, Luke K. T. Univ. of Minneapolis (Mnpls-St. Paul) 1 R01 CA 38932-01	Carcinogenesis of Butylated Hydroxyanisole
31.	LONGNECKER, Daniel S. Dartmouth College 1 R01 CA 38131-01	Glyceryl Monooleate and Pancreatic Carcinogenesis
32.	LUDLUM, David B. Albany Medical College 5 R01 CA 32446-03	Repair of Carcinogenic Lesions in DNA
33.	MARNETT, Lawrence J. Wayne State University 5 R01 CA 32506-03	Cancer Chemoprevention and Arachidonate Metabolism
34.	McCORMICK, Anna M. University of Texas Health Science Center (Dallas) 5 R01 CA 31676-03	Metabolism of Chemopreventive Retinoids
35.	McCORMICK, David L. IIT Research Institute 5 R23 CA 30646-03	Interactions Among Modifiers of Mammary Carcinogenesis
36.	McCORMICK, J. Justin Michigan State University 5 R01 CA 32490-03	Inhibition of Carcinogen--Transformation of Human Cells
37.	MEDINA, Daniel Baylor College of Medicine 2 R01 CA 11944-13	Biology of Mammary Preneoplasias
38.	MEDINA, Daniel Baylor College of Medicine 5 R01 CA 32473-03	Selenium Inhibition of Mouse Mammary Tumorigenesis

	Investigator / Institution / Grant	Title
39.	MEHTA, Rajendra G. IIT Research Institute 5 R01 CA 34664-03	Hormone and Retinoid Interaction in Mammary Tissue
40.	MOORE, Malcolm A. Sloan-Kettering Inst. for Can. Res. 5 R01 CA 32516-03	Mechanisms of Biological Prevention of Leukemogenesis
41.	NAPOLI, Joseph L. University of Texas Health Science Center 5 R01 CA 32474-02	Determinants of Vitamin A Homeostasis
42.	NILES, Richard M. Boston University 2 R01 CA 32543-03	Regulation of Growth and Differentiation by Retinoids
43.	ONG, David E. Vanderbilt University 2 R01 CA 20850-08	Cancer and Vitamin A
44.	PROUGH, Russell A. University of Texas Health Science Center (Dallas) 5 R01 CA 32511-03	Inhibitor Effects on Monooxygenase Function
45.	REDDY, Janardan K. Northwestern University 5 R01 CA 32504-03	Antioxidants and Peroxisome Proliferator Carcinogenesis
46.	REINERS, John J., Jr. University of Texas Sys. Can. Ctr. 1 R01 CA 34469-02	Inhibition of Chemical Carcinogenesis by Interferon
47.	ROGERS, Adrianne E. Massachusetts Institute of Technology 5 R01 CA 32520-02	Anticarcinogenic Effects of Selenium and Vitamin A
48.	ROGERS, Adrianne E. Boston University 7 R01 CA 39222-01	Azaserine Carcinogenesis--Effects of Methionine, Choline
49.	RUDDLE, Nancy H. Yale University 5 R01 CA 32447-03	Lymphotoxin and Interferon Inhibition of Carcinogenesis
50.	SHERTZER, Howard G. University of Cincinnati 1 R01 CA 38277-01	Chemoprotection from N-Nitrosamines by Dietary Indole
51.	SLAGA, Thomas J. University of Texas Sys. Can. Ctr. 5 R01 CA 34521-03	Inhibition of Tumor Promotion by Antioxidants

52.	STEINBERG, Mark L. New York University 5 R01 CA 32485-03	Effects of Retinoids on Human Epidermal Keratinocytes
53.	THOMPSON, Henry J. University of New Hampshire 5 R01 CA 32465-03	Breast Cancer Chemoprevention and Polyamine Biosynthesis
54.	THOMPSON, Henry J. University of New Hampshire 1 R01 CA 38265-01	Cancer Prevention and Vanadium
55.	VERMA, Ajit K. University of Wisconsin (Madison) 1 R01 CA 36323-01A1	Inhibition of Skin Tumor Promotion
56.	WALL, Monroe E. Research Triangle Institute 1 R01 CA 38245-01	New Natural and Synthetic Inhibitors of Carcinogenesis
57.	WANG, Alexander Y. University of Texas Sys. Can. Ctr. 1 R01 CA 35363-01A1	Vitamin E and Cancer
58.	WATTENBERG, Lee W. Univ. of Minnesota (Mnpls-St. Paul) 5 R01 CA 14146-12	Inhibition of Carcinogenesis by Phenols and Thiols
59.	WATTENBERG, Lee W. Univ. of Minnesota (Mnpls-St. Paul) 1 R01 CA 37797-01	Diterpenes as Inhibitors of Carcinogenesis
60.	WEBB, Thomas E. Ohio State University 1 R01 CA 38125-01	Beta-glucuronidase Inhibition and Chemical Carcinogenesis
61.	WIEBEL, Friedrich J. GSF-Research Center Gesellschaft fur Strahlen 5 R01 CA 32541-02	Carcinogen Inactivation by Conjugation with Glutathione
62.	WOLF, George D. Massachusetts Institute of Technology 5 R01 CA 13792-08	Vitamin A and Glycoproteins of Skin Tumors

CONTRACTS ACTIVE DURING FY 85

Investigator/Institution/Contract Number	Title
63. COHEN, Leonard A. American Health Foundation N01-CP-05722	Dose Response Studies on Phenolic Antioxidants (Mammary Gland)
64. DAWSON, Marcia I. SRI International N01-CP-05600	Synthesis of New Retinoids for the Chemoprevention of Epithelial Cancer
65. HICKS, R. Marian Middlesex Hospital Medical School N01-CP-05602	Chemoprevention of Epithelial Cancer by Retinoids (Bladder)
66. HILL, Donald L. Southern Research Institute N01-CP-41005	Studies on Toxicology and Pharmacology of Biological and Chemoprevention Agents
67. MCCORMICK, David L. IIT Research Institute N01-CP-41063	Studies of Toxicology and Pharmacology of Biological and Chemopreventive Agents
68. MOON, Richard C. IIT Research Institute N01-CP-05718	Chemoprevention of Epithelial Cancer by Retinoids (Mammary Gland)
69. REDDY, Bandaru S. American Health Foundation N01-CP-05721	Dose Response Studies on Phenolic Antioxidants (Intestinal Tract Model)
70. WILLIAMS, Gary M. American Health Foundation N01-CP-05723	Dose Response Studies on Phenolic Antioxidants (Liver)

SUMMARY REPORT

CARCINOGENESIS MECHANISMS

The Carcinogenesis Mechanisms component of the Branch consists of studies relating to the metabolism, toxicity, physiological disposition, and mechanisms of action of carcinogens and their metabolites. Studies involving the synthesis of both known and suspect carcinogens or the development of derivatives for molecular structure-activity relationships are also included. Other studies deal with the identification of reactive metabolites and the isolation and characterization of carcinogen metabolizing enzymes. Currently the research grant is the sole instrument of support for this area. In FY 1985 there was one program project grant (P01), one young investigator grant (R23), one small business innovation research grant (R43), one conference grant (R13), and 92 traditional research grants (R01) with a total support funding of $9.57 million.

Grants Activity Summary

The grants in the program are most easily classified by the agent under study. Approximately half are concerned with studies of polycyclic aromatic hydrocarbons (PAH), alkylating agents and aromatic amines. The remainder deals with studies involving many carcinogens from more than one of the above-mentioned groups.

Polycyclic Aromatic Hydrocarbons: This area remains very active. The oxidation of aromatic hydrocarbons is a subject of intensive investigation because many members of this class of compounds exhibit toxic, mutagenic, or carcinogenic properties. The polycyclic aromatic hydrocarbons, acenaphthene and acenaphthylene, are found as constituents of coal tar, tobacco smoke, and as organic contaminants of ground water. Both compounds have been classified by the U.S. Environmental Protection Agency as priority pollutants. To date there have been relatively few studies on the metabolism of these compounds by microorganisms. One project (23) is studying the oxidation of acenaphthene and acenaphthylene by a bacterium, *Beijerinckia* sp. The proposed pathways of acenaphthene and acenaphthylene oxidation by the *Beijerinckia* sp. both lead to the formation of acenaphthenequinone as the final end product of metabolism. However, the routes taken from these compounds to the common end product appear to be considerably different. Based upon metabolite profiles, the oxidation of acenaphthene is reported to proceed through two successive monooxygenations and two dehydrogenation steps, whereas acenaphthylene oxidation appears to proceed through an initial dioxygenation step, which is catalyzed by a dioxygenase enzyme system, followed by two dehydrogenation steps. Whether acenaphthene oxidation is catalyzed by a mono- or dioxygenase enzyme system will be the subject of further investigation.

Some polycyclic hydrocarbons are potent carcinogens in rodent bioassays; others have only weak or no carcinogenic activity. However, many PAH with little or no carcinogenic activity either increase or decrease the carcinogenic activity of others. To determine the effects of the coadministration of benzo(e)pyrene (BeP) on the formation of specific 7,12-dimethylbenz(a)anthracene (DMBA)-DNA adducts as well as the total binding of DMBA to DNA, Syrian hamster embryo cell cultures were exposed to DMBA and BeP at ratio of (1:3) and (1:15) for 24, 48 and 72 hours (2). At the high BeP dose (1.5 microgram g/ml medium) the formation of three major adducts designated P1, P2, P3 decreased 3-4 fold at all times studied. In contrast, in cells exposed to the low BeP dose (0.3 microgram g/ml medium) the

amount of both the anti-DMBA-DNA adducts (P1, P3) increased, and the amount of the syn-DMBA-DNA adduct decreased at 48 and 72 hours. These investigators found that low doses of BeP specifically enhanced the formation of anti-DMBA-diol-epoxide DNA adducts which increased the total binding of DMBA to DNA while high doses decreased the formation of all adducts and hence the total binding to DNA. The reduction in DMBA initiation by BeP may depend upon the ratio of BeP to DMBA exposure and low ratios of BeP to DMBA might result in an increase in initiation of DMBA similiar to that induced by BeP and BaP (benzo(a)pyrene). These results suggest that hydrocarbons that are potent metabolism inducers may actually serve as inhibitors of carcinogenic hydrocarbon metabolism and activation if coexposure takes place while the poor inducers selectively alter carcinogenic hydrocarbon activation.

Current evidence suggests that covalent modificaton of DNA plays a central role in the mechanism of tumor initiation by diverse classes of carcinogenic agents including PAH. However, it remains to be determined which DNA-adducts are most important; whether DNA-adducts are removed efficiently or inefficiently thus introducing errors in the DNA; or whether DNA-adducts must persist in the DNA for long periods of time for tumor initiation. Two grants are conducting research where the early critical events in PAH carcinogenesis in mouse skin as a specific target tissue are being investigated. In one grant, the interaction of (+)anti-benzo(a)pyrene-7,8-diol-9,10-epoxide ((+)anti-BPDE) and (-)anti-BPDE with the various subpopulations of epidermal cells from SENCAR mice is being compared with the binding of (+)anti BPDE and (-)anti BPDE with epidermal nuclear proteins of SENCAR mice (83). The other grant is examining, in detail, the time course of total covalent binding of BaP to mouse epidermal DNA in vivo over a 21-day period. Covalent binding of BaP to epidermal DNA reached a peak at 24 hours after treatment. Between 24 and 48 hours after application of the hydrocarbon there was a very rapid drop in the level of bound BaP to a value about 50% of the maximum level at 24 hours. Thereafter, the level of bound BaP disappeared at a much slower rate (11). These investigators have also examined the time course of formation and disappearance of specific BaP DNA-adducts during the first 7 days of the 21-day time course. The results indicated that all of the BaP DNA-adducts disappeared from epidermal DNA with a biphasic decay curve similar to that observed for total binding. However, not all of the BaP DNA-adducts disappeared from epidermal DNA at the same rate. The most consistent observation was that the formation and disappearance of those DNA-adducts formed with deoxyadenosine was more rapid than the major adduct (+)anti-BPDE-deoxguanosine. The results suggest that epidermal cells have the capacity to repair BaP DNA adducts but this may be limited by the heterogeneous nature of cells within the epidermis. The time course of total covalent binding of DMBA to mouse epidermal DNA has also been analyzed by this group. Like BaP DNA-adducts, DNA-adducts derived from DMBA also reached a maximum level at 24 hours and disappeared with a biphasic decay curve. The half-lives of disappearance of DMBA DNA-adducts during the two phase of decay were similar to those found for BaP DNA-adducts. Analysis of the rate of formation and removal of individual DMBA DNA-adducts suggests that the two major deoxyadenosine adducts disappear more rapidly than the major anti-3,4-diol-1,2-epoxide-deoxyguanosine adduct.

Methylbenz(a)anthracenes (MBAs) have been found in cigarette smoke condensates, in stack gas emissions, and roofing tar extracts, and are identified as potentially environmental hazards to man. One project is evaluating the molecular basis for the carcinogenic differences of the 12 MBAs (96). These investigators stated that the 8,9-double bond of benz(a)anthracene (BA), monomethyl-BA, and 7,12-dimethyl-BA

is the major site of oxidative metabolism. Previous studies of this laboratory indicated that an 8,9-dihydrodiol can be formed at the 8,9-double bond of 8-methyl-BA by rat liver microsomal enzymes. They predicted that a hydroxymethyl-substituted double bond may also be subjected to oxidative metabolism. A compound selected for testing this hypothesis was 8-hydroxymethyl-BA. As they predicted, an 8,9-dihydrodiol was found to be a metabolite of 8-hydroxymethyl-BA. The experimental results obtained in this laboratory have established that oxidative metabolism may occur at either a methyl- or a hydroxymethyl-substituted double bond of PAH. In another paper, the same group has reported that optically active metabolites are formed during the in vitro and in vivo metabolism of PAHs due to the stereoselective properties of the microsomal mixed-function oxidases, epoxide hydrolase, and other drug-metabolizing enzymes. These investigators developed a rapid and sensitive chiral stationary phase (CSP) HPLC method that directly resolves some enantiomeric derivatives of BA and BaP. With this CSP-HPLC method, they have determined enantiomeric compositions of some dihydrodiol metabolites.

The importance of epoxide hydrolase(s) in determining the deposition of active epoxide intermediates of numerous chemical carcinogens has long been recognized. It has often been erroneously assumed that the enzyme exists in a single membrane-bound form. An attempt is being made in one project to demonstrate that at least one other unique major form of microsomal epoxide hydrolase exists (32). Both the membrane-bound epoxide hydrolase (EH1) and the cytosolic epoxide hydrolase (EH2) have been identified from human lung and liver by the group. They noted that the catalytic properties of these enzymes are very similar to their counterparts from mice and guinea pigs, as demonstrated by their Km values and susceptibility to inhibition by styrene oxide, trichloropropene oxide, cyclohexene oxide, 4-phenylchalcone oxide, and 4'-phenylchalcone oxide. Significant progress has been made by this group on the production and isolation of monoclonal antibodies to EH1. Two fusions have yielded 22 monoclonal antibody-producing cell lines. The antibodies produced by these lines have been tested and classified according to immunoglobulin subtype, and each cell line is producing a unique antibody to EH1.

Alkylating Agents: A great deal of recent evidence suggests that nitrosamines are activated to ultimate carcinogens by a set of sequential and perhaps competing biochemical transformations. One group recently has elucidated the characteristics of a chemical model reaction which cleaves a beta-hydroxynitrosamine to a small fragment nitrosamine (51). This process could involve enzyme initiated oxidation to the aldehyde. Beta-nitrosamino aldehydes transfer their nitroso group to other amines with ease and will deaminate primary amines. It is obvious that the occurrence of these molecular events within the cell could result in somatic mutation and cell change. Their further chemical investigation of the transnitrosation reaction of beta-nitrosamino aldehydes has shown that the nitroso transfer results from an intermediate formed from the amine and the aldehyde. The substrate aldehydes do not release free nitrite or nitrous acid into the media. The reaction of a primary or a secondary amine with a beta-nitrosamino aldehyde not only involves transnitrosation to that amine but the formation of an diimine of glyoxal. Glyoxal is known to be mutagenic and react preferentially with the guanine bases in nucleic acids. It is possible therefore that beta-nitrosamino aldehyde acts by both deamination and formation of glyoxalamines which could bind to RNA or DNA. This group, through collaboration with the University of Minnesota, has also investigated the possibility of high tumor incidence in an industry in the Minneapolis-St. Paul area. They studied samples of metal working fluids and found that they contain both direct acting and indirect acting mutagens. The manufacturer of the fluid indicated the presence of about 2%

N-nitrosodiethanolamine (NDELA) in the fluid. They found the samples to contain about 12% nitrite and suggested this as the source of the direct acting mutagen. The metal working fluid samples were treated to remove nitrite and other anions and resubmitted for further mutagen assay. Removal of the anionic components eliminated the direct acting mutagenic properties of the fluids. While numerous laboratory studies have shown that carcinogenic nitrosamines can be formed from the aqueous nitrosation of environmentally prevalent tertiary amines, there is accumulating evidence that the pathway from tertiary amine to nitrosamine is branched and less well understood than once thought. This contention is supported by recent findings in another project (52) which have demonstrated the extremely rapid formation of dimethylnitrosamine from a tertiary amine at 25°. Rapid nitrosamine formation from tertiary amines appears to be associated with substrates containing electron rich aromatic rings, according to these investigators.

Several grants support research on metabolism and mechanisms of action of N-nitroso-compounds. One project is studying the metabolism of various N-nitroso drugs and related compounds in a variety of animals (54). N-nitrosomethylaniline (NMA) induces tumors of the esophagus selectively in rats after repeated subcutaneous injection. The distribution in the body, rate of disappearance from organs, tissues, and blood, and excretion in the urine of NMA were investigated after various single doses were given IP to rats. The compound was distributed fairly evenly throughout the body with no preferential concentration in the esophagus, its target organ for carcinogenicity. The mechanism by which unsymmetric dialkylnitrosamines such as methyl-n-amylnitrosamine (MNAN) induce esophageal cancer in rats is being studied in another project (60). Their hypothesis, based on studies in the pancreas and bladder, is that nitrosamines such as MNAN are converted in the esophagus to active metabolites still bearing the nitrosamine group, e.g., beta-oxo-alkyl derivatives, which are readily alpha-hydroxylated to give alkylating agents. Supporting this view, MNAN was converted in vitro by rat esophagus into nitrosamine metabolites that contained hydroxy and keto groups.

Under the mechanisms of action designation, the ability of several N-nitroso compounds to alkylate cellular macromolecules, particularly DNA and RNA, is being investigated. In one project, the principal investigator is studying the mode of action of certain nitrosamines and the extent of DNA methylation by the nitrosamines in the component cells of the pancreas (1). In one program project the investigators are studying what role nitrite and nitroso compounds play in the etiology of human cancer (87). Toward this end, a number of tools and experimental systems have been developed which are capable of measuring the flux of precursors and the quantity of endogenous synthesis of compounds related to nitroso carcinogens in man. They also study the amount and types of N-nitrosamino acids excreted by humans in urine and on the modulation of formation of nitroso compounds in the ferret and dog stomach. In addition to excreted nitrosamines they measure the flux of methylamines and their endogenous synthesis from precursors such as choline. In the area of carcinogenic precursors they investigate the chemistry of indoles as potential gastric mutagens and carcinogens. They have identified factors in gastric juice which promote nitrosation and naturally occurring substances which inhibit nitrosation.

Aromatic Amines: Various aspects of the chemistry, metabolism, and mechanisms of action of this class of carcinogens are subjects of this subgroup of grants. Mammary carcinogenesis by N-substituted aryl compounds is one of the important projects (55). These investigators discovered that mammalian peroxidases such as

lactoperoxidase (bovine milk) and extracts of rat uterine or mammary gland peroxidative activities in the presence of H_2O_2 and Br^- catalyzed oxidation of the carcinogen, N-hydroxy-N-2-fluorenylacetamide (N-OH-2-FAA) to 2-nitrosofluorene (2-NOF) which is a potent direct mutagen. This reaction differs from the oxidation by horseradish peroxidase/H_2O_2 in which nitroxyl free radical is formed from N-OH-2-FAA yielding equimolar amounts of 2-NOF and N-acetoxy-2-FAA. Their main objectives are to elucidate the mechanism of conversion of N-OH-2-FAA to 2-NOF by peroxidase/H_2O_2/Br^- systems and establish its occurrence in vivo as well as potential relevance in tumorigenesis.

The role of metabolism in the biliary excretion of drugs is the subject of another grant (48). The investigators have shown in a rat hepatic microsomal system that glutathione (GSH), at physiological concentrations, markedly stimulates both oxidative pathways (N-demethylation and ring-hydroxylation) of the azo dye, dimethylaminoazobenzene. In studying the mechanism of this phenomenon, they demonstrated that two other SH compounds, 2-mercaptoethanol and dithiothreitol, also stimulated ring-hydroxylation. However, unlike GSH, both depressed N-demethylation at all concentrations used. Thus, the stimulatory effect of GSH was not simply a nonspecific SH effect, but was somewhat more selective in nature.

Genetically controlled differences in N-acetylation have been implicated in susceptibility to drug-induced urinary bladder cancer resulting from exposure to aromatic amine carcinogens. There is evidence that rapid and slow acetylators differ in the amount of DNA damage that is elicited by some chemicals that are N-acetylated. Susceptibility to the toxic effects of xenobiotics can be determined by genetic factors. The genetically controlled acetylator polymorphism offers a well-defined system in which to investigate the relationship between differences in biotransformation and chemical toxicity. The principal investigator of one grant (58) is evaluating the capacity of primary hepatocyte cultures to N-acetylate and to reflect the genetically determined acetylator polymorphism. The cytotoxic and genotoxic potential of compounds that are metabolized by N-acetyltransferase is also being determined. Carcinogenic N-substituted aryl compounds were initially regarded with concern primarily because of exposure due to their industrial use. More recently it has been recognized that man is exposed to structures with similar features and biological activities as the consequence of the pyrolysis of food, pharmacological intake and environmental contamination with by-products of combustion. The molecular events that are responsible for the induction of tumors by N-substituted aryl compounds is the subject of another grant (44). In order to better define the biochemical mechanisms by which N,O-acyltransferase activates chemical carcinogens to reactive states, the investigators purified and characterized the rat liver arylhydroxamic acid N,O-acyltransferase. Successful purification of this enzyme will allow the investigation of several critical steps in the activation of arylhydroxamic acids to reactive carcinogens.

Hormones: Another important research activity reported in the area of carcinogenesis mechanism studies is hormonal carcinogenesis. Interest in this area has accelerated in recent years because of many new developments in the biochemistry of hormones, drug related activities, and newer developments in in vitro and in vivo studies.

Over the last decade compelling evidence has accumulated which indicates a causal link between estrogens and a variety of human cancers. Based on experimental studies (49), it was concluded that there are hormonal and carcinogenic aspects

for estrogens, both natural and synthetic, which are involved in renal tumorigenesis in the hamster. Hormonal aspects related to this tumor system are based on the presence of specific estrogen receptors in the untransformed kidney which increase in number following prolonged estrogen treatment. Moreover, antiestrogens, which inhibit estrogen receptor-complex binding activity, completely block renal tumor induction by estrogens. Finally, estrogens induce progesterone receptors in the hamster kidney and this induction can be inhibited by androgens and antiestrogens. An antiestrogenic effect for a metabolite of estradiol, estriol, was also observed by another group (93) when they studied the effect of estrogens on DMBA induced breast tumors and found evidence for the anti-carcinogenic activity of estriol when this hormone is administered to 50-55 day old Sprague Dawley rats prior to treatment with DMBA.

One of the important epidemiologic observations concerning breast cancer is that early pregnancy confers a lowered lifelong breast cancer risk. Because of the distinct limitations of epidemiologic studies, it has not been possible to determine the mechanism of this effect. Preliminary laboratory studies (82) appear to show that pregnancy confers complete protection against mammary carcinogenesis in rats. This observation provides a valuable phenomenologic model with which to test possible mechanisms to explain the protective effect of pregnancy against mammary carcinogenesis. Most recent results indicate that full-term pregnancy and delivery is more effective than early termination of pregnancy in inducing refractoriness of the mammary gland to carcinogenesis.

Another focus has been on the study or the mechanism whereby estrogenic hormones regulate prolactin gene expression (29). Last year it was reported that an in vitro nuclear transcription system had been set up and estrogen could be shown to have a marked effect on prolactin gene transcription. This work is now in press in Endocrinology. This work was extended to show that estrogen effects on prolactin gene transcription are probably biphasic and involve at least two mechanisms. First, it appears that occupied estrogen receptors are essential for, and closely correlated with, transcription. This response is not effected by inhibitors of protein synthesis suggesting that it is a primary response to the hormone. When so-called "weak estrogens" (estrogens that dissociate from receptors rapidly) are administered, prolactin gene transcription is increased but only transiently. However, after 3 to 4 hours it increases again in a relatively stable manner. This second phase of response is blocked by cycloheximide suggesting it is a secondary response-dependent upon protein synthesis. Studies are now focusing on the involvement of Dopamine in this second phase of response. These investigators were able to show that in cell cultures of pituitary cells estrogen also increases at least in the first phase of transcription. They have continued to study the chromatin structure of the prolactin gene. The initial work on mapping the methylation state and nuclease sensitivity of the gene in pituitary cells is now in press in the Journal of Biological Chemistry. These studies show that the whole prolactin gene domain that has been cloned is in a decondensed and DNase sensitive structure. In contrast, in liver chromatin, the prolactin gene is not DNase sensitive even though some estrogen receptors are present in that tissue. Methylated cytidine in DNA is usually associated with genes that are not being expressed and hypomethylation is correlated with a gene's expression. Methylation of the prolactin gene follows this same pattern and within the coding sequences there is hypomethylation of deoxycytidine. In the flanking regions, which are DNase sensitive, cytidines appear to be fully methylated. Hypersensitive regions have been found in the 5' flanking region of the

chromatin of the prolactin gene in pituitary tissue. These hypersensitive regions are now being sequenced since they appear to be in areas not previously sequenced.

How hormones control growth and the expression of differentiated function of the normal mammary gland, and how these regulatory mechanisms have deviated in hormone dependent breast cancer are the major goals of another grant (75). Specific emphasis was placed upon studying prolactin regulation of milk protein gene expression in normal and transformed mammary cells as a unique model system for understanding peptide hormone action or gene expression. During the past year the fine structure analysis of members of the rat casein gene family was completed. The entire structure of the beta-casein gene has been elucidated and a model of the evolution of the casein family was proposed. Successful transfection of both human T47-D and rat DMBA-induced mammary tumor cells has been accomplished. Studies have revealed that the gamma-casein gene has weak promoter activity, no apparent enhancer activity, and in preliminary experiments the fusion gene is regulated, albeit to a limited extent, by glucocorticoids, but not prolactin. In addition a number of genomic subclones isolated from the beta- and gamma-casein genes, containing exon and limited intronic sequences have been prepared. These subclones were used as probes for analysis of the primary transcripts of the casein genes.

The role of sex hormones in hepatocarcinogenesis in the infant mouse was studied by another grantee (90). They induced focal hepatocellular lesions by a single carcinogenic dose of diethylnitrosamine (DEN), and studied the effect of gonadectomies on the development of these foci. The key observation was that the change in the hormonal environment did not simply change the slope of the focal growth rate, but rather that it triggered three separate events: first, the reduction in the average focal volume (regression); second, the cessation of focal growth; and, third, the resumption of focal growth. Thus it appears that sex hormones can modulate the degree of cell proliferation (triggered by the carcinogen) by influencing selectively the growth of the hormone-responsive cells and thus delaying their progression to hormone-unresponsive cell populations and carcinomas.

Like two other sites of cancer, the breast and the prostate gland, the endometrium is an end organ which is highly sensitive to stimulation by gonadal steroids. The role of endogenous estrogen metabolism in the development of endometrial cancer was investigated by another group (42). They conducted studies to evaluate circulating estrogen levels in serum from postmenopausal women with or without endometrial cancer. Using a rat model, these investigators examined the in vivo transport of all the types of estrogen preparations currently available commercially for replacement therapy to some of the known sites of estrogen action. These included estradiol, estrone, ethinyl estradiol, estrone sulfate and diethylstilbestrol. For all estrogens tested, the percentages of estrogens available for transport from human post menopausal sera into the liver greatly exceed that which were available to the brain and uterus. This here-to-fore unrecognized mechanism may help explain the enhanced hepatic versus non-hepatic effects of estrogen replacement.

Several reports have indicated that progestins can produce histological changes in endometrial adenocarcinoma which are characteristic of the transition from proliferative to secretory endometrium, namely, formation of subnuclear vacuoles, corresponding to glycogen deposition, and a generally quiescent appearance of the

epithelial cells, characterized by an absence of mitoses and low nuclear-cytoplasmic ratio. In another grant (33) the "in vitro" responsiveness of endometrial adenocarcinoma to progestins was evaluated histologically by incubation of tissue fragments in medium containing 10^{-6}M methoxyprogesterone acetate. The findings suggest that simple organ culture and histological procedures can be used to identify specimens of endometrial cancer that have functional progesterone receptors and are capable of responding to progestins. In addition, in vitro responses to progestins may indicate the presence in endometrial cancer tissue of functional estrogen receptors and potential responsiveness to antiestrogens, since estrogen stimulation appears to be needed for the synthesis of progesterone receptors.

It has been known for many years that perinatal exposure to estrogens causes reproductive dysfunction and abnormalities of the reproductive tract. In another grant (6), investigators have examined the estrogen-induced responses of uterus and vagina in the normal rat and compared them to those observed in animals which have received estrogen during the neonatal period. In addition, estrogen induced responses of uterus and vagina also were examined during the neonatal period to detect specific patterns of response induced by such estrogenic agents as diethylstilbesterol, kepone and T-chlorophenol, O-chlorophenol-trichloroethane. These attempts to develop a screening test for neonatal carcinogenesis, which may be specific for a particular compound, is of considerable importance. There are no current methods available for detection and prediction of hormonally active agents which cause reproductive dysfunction and abnormalities.

Murine dorsolateral prostate has been shown to undergo estrogen and chemical carcinogen-induced neoplastic transformation. A group of investigators (67) found that testosterone and estrogen-administration to Noble rats induce hyperplastic and dysplastic changes in the dorsolateral prostate which closely resemble preneoplastic and carcinomatous lesions of the human prostate. By successful manipulation of the in vitro environment, they established a viable, long-term (22 day) culture of C_3H mouse prostate and explants of Noble rat dorsolateral prostate for 12 days in culture. With these organ culture systems they will test the concept that sex hormones promote the action of chemical carcinogen by modulating prostatic basal-cell proliferation and epithelial differentiation.

Steroid hormones, in general, effect their biologic responses in target tissues through the mediation of high affinity, specific binding proteins, called receptors, which are present in unique amounts in such responsive tissues. The role of specific intracellular receptor proteins in the estrogenic regulation of growth and function in reproductive tissues and cancers is the objective of another study (30). A combination of biochemical, immunochemical and recombinant DNA techniques are being used to determine the structure, composition and intracellular dynamics of the estrogen receptor from calf, rat and human sources. A major accomplishment during this period has been the use of monoclonal antibodies to develop immunoassays for estrogen receptors that can quantitate and localize receptors in normal and neoplastic target cells. Two clinically useful assays have been perfected, in collaboration with Abbott Laboratories: an immunocytochemical assay (ER-ICA) for determining the distribution of receptor-containing cells in a cancer biopsy, and an enzyme immunoassay (ER-EIA) for measuring the receptor content of tumor extracts. Both assays are being used to assess the prognosis and probable response to endocrine therapy of estrogen-dependent reproductive cancers. The ER-EIA, which is now commercially available from Abbott

as a research kit, has been used to evaluate more than 500 breast cancer cytosols in field trials at several sites in the U. S. and in Europe.

Investigators over the last several years have determined the role of 1,25 dihydroxyvitamin D_3 (D_3) in intestinal calcium transfer and bone readsorption. This process is believed to proceed through a hormone-receptor mechanism in which a high affinity intracellular receptor mediates hormonal effects through the initiation of gene transcription and translation. The hormone D_3 is an essential element in the homeostatic regulation of calcium and phosphorous. A considerable body of evidence suggests that D_3 acts by inducing the de novo synthesis of transport proteins and that the intracellular mechanism of action of D_3 is similar to other steroid hormones. Receptors for D_3 have been identified in over twenty target organs including intestine, bone, kidney, pancreas, skin, mammary tissue, pituitary and chorioallantoic membrane. A recent study (80) has explored the hypothesis that D_3 is a regulatory factor for growth of malignant cell lines derived from tumors arising in D_3 responsive tissues. It was found that D_3 receptors exist in malignant cells. The cell lines studied provide an excellent tool to determine whether D_3 actions on malignant cells is via a control of cellular calcium handling. In addition, it was found that HPLC gel exclusion of D_3 receptors from malignant cells provided a rapid identification of specific receptors.

Other Agents: The sub-grouping of this class consists of grants investigating a variety of agents not in the other sub-categories or with mixtures of agents from more than one category. One project has been concentrating on two areas. The first is an investigation of the stereochemistry of certain enzyme reactions. This is done by structural analysis of enzymes, substrates, and inhibitors both individually and as complexes. The second is an investigation of the structures of some polycyclic mutagens, carcinogens and their metabolic products, and molecular complexes of such compounds (24). These investigators employed x-ray crystallographic techniques to study some acridine, benz(a)anthracene and chrysene derivatives that are frameshift mutagens, carcinogens and/or antitumor agents.

The goal of another grant has been to elucidate the biochemical mode of action of the hydrazine ingredient of cultivated mushrooms (89). They reported that the mushroom of commerce, Agaricus bisporus, is known to contain beta-N-(gamma-L(+)glutamyl)-4-hydroxymethylphenylhydrazine (synonym agaritine). Agaritine has been found to break down by gamma-glutamyltranspeptidase, an enzyme present in the mushroom as well as in humans, to 4-hydroxymethylphenylhydrazine (HMPH) and 4-(hydroxymethyl)benzenediazonium ion (HMBD). In addition, HMPH undergoes further changes in vitro and yields 4-methylphenylhydrazine (MPH). These investigators have demonstrated the carcinogenicity of HMPH, HMBD and MPH given as salts to mice, while the administration of Agaritine yielded negative results.

Thiono-type compounds containing =P(S)- or -C(S)- linkages are widely used as pesticides, drugs, and industrial materials, despite mutagenic and carcinogenic hazards. One project is studying the reason(s) for the carcinogenicity and/or mutagenicity of compounds containing =P(S)- and -C(S)- linkages by simulating biological oxidations and studying the stability, structure, and reactions of culpable species with functional groups likely to be involved in bio-macro-molecular damage (16). They will extend the structure-activity relations to permit prediction of relative hazards to the public and to provide leads from carcinogens considered as possible antineoplastics, so that undesired activity then can be "designed out."

CARCINOGENESIS MECHANISMS

GRANTS ACTIVE DURING FY85

Investigator/Institution/Grant Number	Title
1. ARCHER, Michael C. Ontario Cancer Institute 5 R01 CA 26651-06	Mechanism of Nitrosamine Alkylation of DNA and RNA
2. BAIRD, William M. Purdue University West Lafayette 5 R01 CA 28825-05	Modifiers of Chemical Carcinogenesis in Cell Culture
3. BHARGAVA, Madhu M. Yeshiva University 5 R01 CA 32268-02	Protein Binding in Hepatic Fate of Chemical Carcinogens
4. BUHLER, Donald R. Oregon State University 5 R01 CA 22524-07	Pyrrolizidine Alkaloid Toxicity, Metabolism and Binding
5. CAVALIERI, Ercole L. University of Nebraska Med. Ctr. 5 R01 CA 32376-03	Mechanisms of Mammary Carcinogenesis by Hydrocarbons
6. CLARK, James H. Baylor College of Medicine 2 R01 CA 26112-04A1	Effect of Estrogen on Normal and Abnormal Cell Growth
7. COHEN, Samuel M. University of Nebraska Med. Ctr. 5 R01 CA 32313-03	Non-Mutational Multistage Urinary Bladder Carcinogenesis
8. COLBY, Howard D. West Virginia University 5 R01 CA 22152-06	Adrenal Carcinogen Metabolism
9. COX, Ray Univ. of Tennessee Ctr. Hlth. Sciences 5 R01 CA 15189-11	Ethionine Carcinogenesis
10. CUCHENS, Marvin A. Univ. of Mississippi Med. Ctr. 5 R01 CA 33111-02	Carcinogenesis of B-Lymphocytes Peyer's Patches
11. DIGIOVANNI, John University of Texas Sys. Can. Ctr. 5 R01 CA 36979-02	Role of DNA-Binding in Skin Tumor Initiation
12. EL-BAYOUMY, Karam E. American Health Foundation 5 R01 CA 35519-02	Nitroaromatics: Carcinogenicity and Metabolism

	Investigator / Institution / Grant	Title
13.	ENSLEIN, Kurt Health Designs, Inc. 1 R43 CA 37494-01	SAR Estimation of Carcinogenesis Bioassay Results
14.	ERNSTER, Lars University of Stockholm 5 R01 CA 26261-06	The Metabolism of Polycyclic Hydrocarbons and Cancer
15.	FANNING, James Collier Clemson University 5 R01 CA 35733-02	The Nitrosation of Amines with Iron Nitrates
16.	FIELD, Lamar Vanderbilt University 5 R01 CA 30321-03	Thiono-Type Compounds and Their Relation to Cancer
17.	FLOSS, Heinz G. Ohio State University 1 R01 CA 37661-01	Biochemical Mechanisms of Nitrosamine Carcinogenesis
18.	FLOYD, Robert A. Oklahoma Medical Research Fdn. 5 R01 CA 18591-09	Carcinogen Free Radicals in Arylamine Metabolism
19.	FORD, George P. Southern Methodist University 1 R01 CA 38473-01	The Prediction of Nucleoside-Carcinogen Reactivity
20.	FRANKLIN, Michael R. University of Utah 5 R01 CA 15760-10	Modification of Procarcinogen Enzymatic Activation
21.	GESSNER, Theresa Roswell Park Memorial Institute 2 R01 CA 24127-05A1	Conjugations of Carcinogen Metabolism
22.	FRANTZ, Andrew G. Columbia University 5 R01 CA 11704-14	Studies on Prolactin and Other Peptides
23.	GIBSON, David T. University of Texas (Austin) 5 R01 CA 19078-10	Microbial Degradation of Carcinogenic Hydrocarbons
24.	GLUSKER, Jenny P. Institute for Cancer Research 5 R01 CA 10925-36	Application of Crystallographic Techniques
25.	GOLD, Avram Univ. of North Carolina (Chapel Hill) 5 R01 CA 28622-03	Activation of Polycyclic Environmental Mutagens

26. GOLD, Barry I.
University of Nebraska Med. Ctr.
5 R01 CA 24554-06
Epoxidation in Chloro-Olefin Carcinogenesis

27. GOLD, Barry I.
University of Nebraska Med. Ctr.
1 R01 CA 38976-01
Metabolism and Genotoxicity of Nitrosamines

28. GOLDMAN, Peter
Harvard University
5 R01 CA 34957-03
Carcinogen Metabolism by Host Intestinal Bacteria

29. GORSKI, Jack
Univ. of Wisconsin (Madison)
5 R01 CA 18110-10
Prolactin Synthesis in Normal and Neoplastic Tissue

30. GREENE, Geoffrey L.
University of Chicago
5 R01 CA 02897-29
Steroids and Growth

31. GROVER, Philip L.
University of London
5 R01 CA 21959-08
Mechanisms of Activation of Polycyclic Hydrocarbons

32. GUENTHNER, Thomas M.
University of Illinois (Chicago)
5 R01 CA 34455-03
Toxicologic Implications of Multiple Epoxide Hydrolases

33. GURPIDE, Erlio
Mount Sinai School of Medicine
5 R01 CA 15648-11
Steroid Dynamics in Human Endometrial Cancer

34. GURTOO, Hira L.
Roswell Park Memorial Institute
5 R01 CA 25362-06
Genetics of Aflatoxin Metabolism-Role in Carcinogenesis

35. HARRINGTON, George W.
Temple University
5 R01 CA 18618-10
Electroanalytical Studies of N-Nitrosamines

36. HARVEY, Ronald G.
University of Chicago
5 R01 CA 36097-02
Mechanism of Carcinogenesis of Polycyclic Hydrocarbons

37. HECHT, Stephen S.
American Health Foundation
5 R01 CA 32242-03
Carcinogenic Methylated PAH: Structural Requirements

38. HOLLENBERG, Paul F.
Northwestern University
2 R01 CA 16954-09
Hemoprotein-Catalyzed Oxygenations of Carcinogens

	Investigator / Institution / Grant	Title
39.	HYLEMON, Phillip B. Virginia Commonwealth University 5 R01 CA 17747-11	Bile Acids and Large Bowel Carcinogenesis
40.	JEFCOATE, Colin R. Univ. of Wisconsin (Madison) 2 R01 CA 16265-11	Metabolism of Polycyclic Hydrocarbons and Carcinogenesis
41.	JENSEN, David E. Temple University 5 R01 CA 31503-04	Chemical Decomposition of Alkylating Nitroso Compounds
42.	JUDD, Howard L. Univ. of California (Los Angeles) 5 R01 CA 23093-07	Estrogen and Androgen Studies in Endometrial Cancer
43.	KAUFFMAN, Frederick C. Univ. of Maryland (Baltimore) 5 R01 CA 20807-08	Pharmacology of Carcinogen Activation in Intact Cells
44.	KING, Charles M. Michigan Cancer Foundation 5 R01 CA 23386-08	Mechanistic Approaches to Carcinogenesis
45.	KOREEDA, Masato University of Michigan (Ann Arbor) 2 R01 CA 25185-07	The Bio-organic Chemistry of Arene Oxides and Related Epoxides
46.	LEE, Mei-Sie Michigan Cancer Foundation 1 R01 CA 37885-01A1	Metabolic Activation of Unsubstituted Hydroxamic Acid
47.	LEHR, Roland E. University of Oklahoma (Norman) 5 R01 CA 22985-09	Diol Epoxide and Other Derivatives of PAH and AZA-PAH
48.	LEVINE, Walter G. Yeshiva University 5 R01 CA 14231-11	Role of Metabolism in the Biliary Excretion of Drugs
49.	LI, Jonathan J. Univ. of Minnesota (Mnpls.-St. Paul) 5 R01 CA 22008-08	Estrogen Carcinogenicity and Hormone Dependent Tumors
50.	LIEHR, Joachim G. Univ. of Texas Hlth. Sci. Ctr. (Houston) 5 R01 CA 27539-05	Mechanism of Estrogen-Induced Renal Carcinogenesis
51.	LOEPPKY, Richard N. University of Missouri (Columbia) 5 R01 CA 22289-08	Nitrosamine Fragmentation and Nitrosamine Carcinogenesis

52. LOEPPKY, Richard N.
University of Missouri (Columbia)
5 R01 CA 26914-06
Carcinogenesis: Nitrosamine Formation and Inhibition

53. LOTLIKAR, Prabhakar D.
Temple University
5 R01 CA 31641-03
Modulation of Mycotoxin Carcinogenesis by Glutathione

54. MAGEE, Peter N.
Temple University
5 R01 CA 23451-07
Formation and Metabolism of N-Nitroso Compounds

55. MALEJKA-GIGANTI, Danuta
Univ. of Minnesota (Mnpls.-St. Paul)
5 R01 CA 28000-06
Mammary Carcinogenesis by N-Substituted Aryl Compounds

56. MANDEL, Richard
Boston University
5 R01 CA 27324-06
Additive and Synergistic Effects of Mutagens

57. MARCHOK, Ann C.
Oak Ridge National Laboratory
5 R01 CA 34137-02
Effects of HCHO and Benzopyrene in A New Tracheal Model

58. McQUEEN, Charlene A.
American Health Foundation
5 R01 CA 33144-03
Genetic Susceptibility to Xenobiotic Toxicity

59. MIRVISH, Sidney S.
University of Nebraska Med. Ctr.
5 R01 CA 32192-02
N-Nitroso Compounds Formed In Vivo from Nitrogen Dioxide

60. MIRVISH, Sidney S.
University of Nebraska Med. Ctr.
5 R01 CA 35628-02
Nitrosamine Metabolism in the Esophagus

61. MORRISON, Harry A.
Purdue University West Lafayette
5 R01 CA 18267-07
Cutaneous Photobiology and Drug Phototoxicity

62. MORTON, Kenneth C.
Michigan Cancer Foundation
5 R01 CA 32303-03
Metabolism of Activation of 4,4'-Met-Bis(2-Chloroaniline)

63. MUKHTAR, Hasan
Case Western Reserve University
1 R01 CA 38028-01
Goeckerman Therapy of Psoriasis--Oncogenic Mechanisms

64. NAGEL, Donald L.
University of Nebraska Med.Ctr.
5 R01 CA 31016-04
An In Vitro Model for Alkylation by Pancreas Carcinogens

65. NEWMAN, Melvin S.
Ohio State University
5 R01 CA 07394-19
Synthesis of Substituted Polycyclic Hydrocarbons

66.	NICOLAOU, Kyriacos C. University of Pennsylvania 5 R01 CA 36196-02	Synthesis and Biology of Unstable Natural Products
67.	OFNER, Peter Tufts University 2 R01 CA 29513-04A1	Androgens in Prostatic and Epididymal Culture
68.	PAQUETTE, Leo A. Ohio State University 5 R01 CA 12115-14	Unsaturated Polyolefins and Hydrocarbon Carcinogenesis
69.	PARTHASARATHY, Rengachary Roswell Park Memorial Institute 5 R01 CA 23704-06	Stereochemistry of Thiol-Disulfide Interchanges
70.	PIETTE, Lawrence H. University of Hawaii at Manoa 5 R01 CA 10977-19	ESR Studies of Biological Free Radical Mechanisms
71.	POUR, Parviz M. University of Nebraska Med. Ctr. 5 R01 CA 35042-02	Prevention of Nasal Cavity Tumors by Castration
72.	PURDY, Robert H. Southwest Fdn. for Biomedical Research 5 R01 CA 24629-06	Mutagenic and Carcinogenic Potential of Estrogens
73.	REINKE, Lester A. Univ. of Oklahoma Health Sci. Ctr. 5 R01 CA 30137-05	Influence of Ethanol on Carcinogen Activation
74.	RIGBY, James H. Wayne State University 5 R01 CA 36543-02	Synthesis of Cocarcinogenic Diterpenes
75.	ROSEN, Jeffery M. Baylor College of Medicine 5 R01 CA 16303-10	Hormonal Regulation of Breast Cancer
76.	SCHECHTER, Joel E. University of Southern California 5 R01 CA 21426-06	Rathke's Pouch-Derived Tumors: Effects of Hormones
77.	SCRIBNER, Norma Pacific Northwest Research Fdn. 5 R01 CA 23712-07	Early and Critical Events in Chemical Carcinogenesis
78.	SEGAL, Alvin New York University 2 R01 CA 24124-05	Carcinogenic Acylating Agents and Mode of Action
79.	SHIMAMURA, Tetsuo Rutgers Medical School 5 R01 CA 30106-03	Mechanisms of Development of Urinary Bladder Cancers

80. SIMPSON, Robert U.
University of Michigan (Ann Arbor)
5 R23 CA 36507-02
Actions of 1,25 Dihydroxyvitamin D3 on Malignant Cells

81. SINCLAIR, Peter R.
Dartmouth College
5 R01 CA 25012-07
Liver Cell Cultures for Study of Carcinogen Activation

82. SINHA, Dilip K.
Roswell Park Memorial Institute
5 R01 CA 36139-02
Protection Against Mammary Carcinogenesis by Pregnancy

83. SLAGA, Thomas J.
University of Texas Sys. Can. Ctr.
5 R01 CA 34962-02
Polycyclic Hydrocarbon Metabolism and Binding in Skin

84. SMITH, Louis C.
Baylor College of Medicine
5 R01 CA 31513-02
Cellular Uptake of Carcinogens

85. STROBEL, Henry W.
Univ. of Texas Hlth. Sci. Ctr. (Houston)
5 R01 CA 37148-02
Colonic Carcinogenesis/Chemotherapy and GI Hormones

86. SULLIVAN, Paul D.
Ohio University (Athens)
5 R01 CA 34966-02
Structure and Metabolism of Substituted Benzo(a)pyrenes

87. TANNENBAUM, Steven R.
Massachusetts Inst. of Technology
5 P01 CA 26731-06
Endogenous Nitrite Carcinogenesis in Man

88. THURMAN, Ronald G.
Univ. of North Carolina (Chapel Hill)
5 R01 CA 23080-08
Pharmacology of Carcinogen Activation in Intact Cells

89. TOTH, Bela
University of Nebraska Med. Ctr.
5 R01 CA 31611-03
Carcinogenesis and Chemistry of Cultivated Mushrooms

90. VESSELINOVITCH, Stan D.
University of Chicago
5 R01 CA 25522-06
Role of Sex Hormones in Hepatocarcinogenesis

91. VOLLHARDT, K. Peter
Univ. of California (Berkeley)
2 R01 CA 20713-08
Activated Mutagenic and Aromatic Hydrocarbons

92. WHALEN, Dale L.
University of Maryland
5 R01 CA 26086-05
Kinetics of Nucleic Acid-Catalyzed Epoxide Hydrolyses

93. WOTIZ, Herbert H.
Boston University
5 P01 CA 28856-05
The Role of Hormones and Binding Proteins in Cancer

No.	Investigator / Institution / Grant	Title
94.	YANG, Chung S. Univ. of Medicine and Dentistry of NJ 5 R01 CA 16788-10	Monoxygenase: Properties and Carcinogen Activation
95.	YANG, Chung S. Univ. of Medicine and Dentistry of NJ 5 R01 CA 37037-02	Enzymology and Mechanisms of Nitrosamine Metabolism
96.	YANG, Shen K. US Uniformed Services University of Health Sciences 5 R01 CA 29133-04	Metabolic Activations of Monomethylbenz(a)anthracenes

SUMMARY REPORT
DIET AND NUTRITION

The Diet and Nutrition component within the Chemical and Physical Carcinogenesis Branch contains 42 grants with funding during FY85 of $3.31 million. The component supports laboratory investigations searching for etiologic factors related to diet, nutrition, and cancer. These investigations include mechanism studies of cancer induction by a variety of dietary constituents (i.e., fats of varying sources and saturation levels, proteins of various types and levels, fiber, nitroso compounds, mycotoxins and other naturally occurring carcinogens, inhibitors of carcinogenesis, compounds associated with the gut including bile acids/fecal steroids and the influence of microflora). In addition, the Program promotes studies which focus on specific dietary factors (i.e., nutrients or micro-nutrients, host factors involved in pathogenesis, and the development of methods or refinements of techniques for identifying putative carcinogens in foods, body fluids or feces, as well as the influence of various methods of food processing and cooking.

The program continues to support several studies on the effects of fat in the diet. One such study (21) was designed to investigate the dose-response effect of 3 levels of dietary corn oil on colon tumor promotion in the rat. In addition, the effect of these diets on fecal bile acids and neutral sterols was assessed since high dietary fat leads to increased levels of secondary bile acids, deoxycholic acid and lithocholic acid, which have been shown to exert promoting effects in colon cancer in rodents.

Weanling female F344 rats were fed a semipurified diet containing 5% corn oil. At 7 weeks of age, all animals except the vehicle (normal saline)-treated rats were given s.c. injections of azoxymethane (AOM; 15 mg/kg body wt., once weekly) for 3 weeks. After 1 week, groups of animals were transferred to semipurified diets containing 13.6 and 23.5% corn oil diets. Fecal bile acids were measured in vehicle-treated rats. All animals were necropsied 34 weeks later and colon tumors were histologically examined for types and number. The body weights of animals fed various levels of corn oil diets were comparable. Animals fed the diets containing 13.6 and 23.5% corn oil consumed about 12 and 8% more food than those fed the 23.5% corn oil diet. Except for the percent of calories from fat in the diet, the intake of total calories, protein, vitamins, minerals and fiber were similar in all dietary groups. Colon tumor incidence (% animals with tumors), and multiplicity (colon tumors/animal) were higher in animals fed 23.5% corn oil diet compared with those fed the diets containing 5 or 13.6% corn oil. Percent of animals with colon tumors: 5% corn oil diet, 67%; 13.6% corn oil diet, 63%, 23.5% corn oil diet, 93%. Colon tumors/animal: 5% corn oil diet, 1.1; 13.6% corn oil diet, 1.2; 23.5% corn oil diet, 1.8. A significant increase in the excretion of several secondary bile acids, namely deoxycholic acid, lithocholic acid, and 12-ketolithocholic acid, and total bile acids was observed in animals fed the diet containing 23.5% corn oil compared with those fed the diets containing 5 and 13.6% corn oil. There was no difference in the excretion of secondary bile acids between the groups fed the 5 and 13.6% corn oil diets.

Another study (22) was designed to evaluate the effects of dietary fats on pancreatic carcinogenesis in the Syrian golden hamster/N-nitroso-bis(2-oxo-propyl)amine(BOP) model. All hamsters were fed the control diet (5% corn oil) when they were initiated with the carcinogen BOP. The experiment was terminated

at nine months instead of the planned 15 months. This was necessary due to the loss of significant numbers of hamsters (total of 52 of 206 hamsters). Nearly all the deaths appear to have been due to "wet-tail disease" which has been described in the hamster. Analysis of the pancreas of the carcinogen-treated hamsters reveals considerably fewer BOP-induced lesions than expected. The major difference between these studies and other major studies which developed the hamster/BOP model is the use of purified diets. The results from this experiment are still being evaluated. A second experiment in this study has been to develop a short-term, rat/azaserine model of pancreatic carcinogenesis. Male Wistar/Lewis rats of 49 days of age were used. All rats were fed the 5% corn oil control diet during the initiation phase. The duration of the experiment was 4 months including a one week initiation phase. The number and size of atypical acinar cell nodules (AACN) per pancreas were evaluated. The major conclusions from these studies are: promotion can be demonstrated in short-term models, and for studies where promotion is to be evaluated, there are considerable savings in carcinogen and time by treating nursing pups.

A third study (16), with the objective of clarifying some of the confusing observations reported in the literature relative to the effect of dietary fat on dimethyl-hydrazine(DMH)-induced colon tumors in rats, is nearing completion. The preliminary observations showed no effect of 24% dietary fat on colon tumorigenesis when low levels of DMH (15 mg/kg, once a week for 5 weeks) were administered i.g. to Sprague-Dawley rats and animals were maintained until moribund or for 60 weeks after receiving the initial dose of carcinogen. Other investigators have used different diets, strains of rats, longer dosing periods and earlier sacrifice schedules. This initial experiment was designed to compare DMH-induced colon tumorigenesis in animals fed 5% or 20% fat diets, using three different diets which varied in micronutrient composition and using the DMH treatment regimen most commonly employed in rodent models.

Weanling animals were allocated to various dietary groups according to the protocol. They were maintained on their respective diets for 3 weeks, then treated once a week with 10 mg/kg DMH via s.c. injection for 20 weeks. Animals received the experimental diets for an additional 10 weeks before sacrifice. Although tumor incidence data are not yet available, it is obvious that the micronutrient composition of the diet has an effect on the animals' response to the toxic effects of DMH. Using weight gain as a criteria, Sprague-Dawley rats were less sensitive than Fischer rats to DMH toxicity. As expected, animals responded poorly to a vitamin deficient diet and did not survive longer than 30 weeks. The most striking differences in diet-carcinogen interactions were seen in the F-344 rats when compared with the Sprague-Dawley rats.

Work is continuing on the effects of dietary factors on UVL-carcinogenesis (4). Previous studies have demonstrated that the degree of dietary lipid saturation influences the expression of UV-induced carcinogenesis. The most recent results demonstrate the influence of dietary lipid level upon UV-carcinogenesis and its modification by antioxidants. Seven hundred twenty female, SKh-HR-1 hairless mice, divided into 16 groups of 45 animals each were employed in the study. The animals received defined diets whose lipid source consisted of 4,2, and 0.75% corn oil. Half of the animals on the respective diets received an antioxidant supplement (2%, W/W) consisting of 1.2% ascorbic acid, 0.5% butylated hydroxytoluene, 0.2% tocopherol, and 0.1% reduced glutathione. Eight groups, representing each of the preceding treatments, served as non-irradiated controls while the remainder received daily, suberythemic UV-radiation until 70 J/cm^2 had been

delivered. Cumulative distribution curves, median tumor times, and tumor multiplicity were determined for each irradiated group. A direct relationship of the cumulative tumor distribution to dietary lipid level was found with 4% lipid level demonstrating the shortest latent period and 0.75% exhibiting the greatest. The tumor distribution plots showed a rather marked influence by antioxidants at the 4% lipid level, diminished at 2%, and was completely negated at the 0.75% lipid level. Median tumor times were 18.4, 19.9, and 21.0 weeks for 4,2, and 0.75% lipid levels, respectively, and 20.6, 21,1, and 20.3 for their respective antioxidant supplemented groups. With regard to tumor multiplicity, the animals exhibited 1.89, 1.30, and 0.61 tumors/animal for 4,2 and 0.75% lipid levels, respectively. Again, only at the two highest lipid levels did significant reductions in tumor multiplicity, due to antioxidants, occur. When lipid peroxidation of epidermal homogenates was examined using thiobarbituric acid (TBA) and peroxide values (PV), TBA values increased upon incubation to 1.99, 1.72, and 0.87 nMol/mg protein for 4,2, and 0.75% dietary lipid levels, respectively. TBA values for the antioxidant supplemented animals remained relatively constant at 0.45 nMol per mg at all dietary lipid levels. PVs were in good agreement with the observed TBA values. These data suggest: (1) that dietary lipid level has a direct effect upon the carcinogenic response, both in regard to tumor latency and multiplicity; (2) antioxidants produce an inhibitory effect almost exactly equal to the degree of exacerbation of carcinogenesis evoked by increasing lipid levels; (3) from examination of both cumulative tumor distribution plots and multiplicity data, it appears that antioxidants produce their inhibitory effects early on in the carcinogenic process and that lipids are able to overwhelm such inhibition, possibly by enhancing promotion events in the carcinogenic continuum; and (4) these data indirectly implicate peroxidative reactions in UV-carcinogenesis and show that antioxidants inhibit such events.

Past studies (17) supported by the Diet and Nutrition component have provided strong evidence that the major mutagen extracted with methylene chloride from a basic aqueous extract of fried ground beef is $MeIQ_x$. Since this finding was reported, significant modifications and improvements have been made in both the extraction and chromatographic procedures. Under these modified conditions a major new peak of mutagenic activity has been observed in Difco beef extract which is less polar than $MeIQ_x$. The new peak was designated I' and a small quantity of it has been highly purified. Work is in progress to obtain NMR and high resolution MS data to give further insight into the structure of this mutagen. If the estimate of the amount of purified material obtained is correct, then this new mutagen will have a higher specific activity than any of the heterocyclic amine mutagens previously isolated from cooked foods.

Addition of proline to fried ground beef prior to frying results in a considerable increase in mutagenic activity. Over the past few years it has been generally observed that about 5-fold increases in mutagenic activity occur in ground beef treated with proline prior to frying. Recently, work in this area makes it appear likely that the increased mutagenic activity in proline-treated fried ground beef is due to the generation of a new mutagen which may not occur naturally in untreated fried beef. Studies are continuing on isolating and identifying this new mutagen.

Work is continuing on dietary minerals. In one experiment (31) the effect of selenium on the process of initiation was investigated using the carcinogens 1-methyl-1-nitrosourea (MNU) and 7,12 dimethylbenz(a)anthracene (DMBA). Preliminary evaluation of the data indicates that selenium was effective in

blocking some aspect of the initiation irregardless of the carcinogen used to induce mammary carcinogenesis. The data are of particular interest since they suggest that selenium can influence tissue responsivity to insult by direct alkylating agents such as MNU. A second experiment, yet to be completed, is a long-term study in which selenium is fed to rats given very low doses of either DMBA or MNU. The palpation data indicate that the protective effect(s) of selenium can be sustained throughout the lifetime of the rat given a small initiating dose of carcinogen. Further comment on these data must, however, await histopathological classification of tumors removed at necropsy.

Significant progress has been made in testing the hypothesis that the effective detoxification of pharmacologic amounts of selenium fed as sodium selenite can, at least in part, account for the chemopreventive effect of selenium via the modulation of the tissue concentrations of the polyamines. It has been possible to demonstrate that feeding pharmacologic amounts of selenite (5 ppm) does result in a 50% reduction in the spermidine concentration in the mammary gland and liver of the female rat. Furthermore, the same response can be duplicated in a primary rat mammary epithelial cell culture system, namely, that supplementation of media with selenite results in a reduction in cell number and a reduction in the cellular content of spermidine with a concomitant increase in the intracellular concentration of putrescine. Studies are now in progress to quantify cellular concentrations of S-adenosylmethionine (SAM) and to determine whether supplementation of culture media with SAM can reverse the effects of selenite on polyamine metabolism.

DIET AND NUTRITION PROGRAM

GRANTS ACTIVE DURING FY85

Investigator/Institution/Grant Number	Title
1. ABRAHAM, Samuel Children's Hospital Medical Center 5 R01 CA 29767-03	Effect of Dietary Fat on Mammary Neoplasia
2. ASANO, Tomoaki University of Notre Dame 5 R01 CA 28276-03	Experimental Carcinogenesis by Dietary Nitrite
3. AYLSWORTH, Charles F. Michigan State University 1 R23 CA 36364-01	Dietary Fat, Cell Communication and Breast Tumorigenesis
4. BLACK, Homer S. Baylor College of Medicine 5 R01 CA 20907-05	Effects of Dietary Factors on UVL-Carcinogenesis
5. BURKE, James P. Pennsylvania College of Podiatric Med. 5 R01 CA 32256-03	Relationship of Zinc to Cellular Membrane Composition
6. CAMPBELL, T. Colin Cornell University 5 CA 34205-02	Dietary Protein and Chemical Carcinogenesis
7. GALIVAN, John H. New York State Department of Health 1 R01 CA 34314-01A1	Vitamin Function in Liver Studied In Vitro
8. GARRETT, Carleton T. George Washington University 5 R01 CA 31324-02	Gene Expression in Nutritionally Promoted Cancer
9. GRUBBS, Clinton J. Southern Research Institute 5 R01 CA 33808-02	Effect of Alcohol on Chemically Induced Cancers
10. HAMILTON, Stanley R. Johns Hopkins University 5 R01 CA 29714-04	Role of Beer and Ethanol in Experimental Colon Cancer
11. HEINIGER, Hans-Jorg Jackson Laboratory 5 R01 CA 19305-06	Cholesterol in Normal and Malignant Lymphocytes
12. IP, Clement C. Roswell Park Memorial Institute 5 R01 CA 27706-06	Selenium Supplement and Dietary Fat in Breast Cancer

	Investigator / Institution / Grant No.	Title
13.	JANGHORBANI, Morteza Boston University 5 R01 CA 38943-02	Dietary Bioavailability of Selenium in Man
14.	LEIGHTON, Terrance J. Univ. of California (Berkeley) 5 R01 CA 36890-02	Origin, Distribution and Control of Mutagens in Wine
15.	MIRVISH, Sidney University of Nebraska Med. Ctr. 5 R01 CA 30593-03	Significance of Nitrosourea Formation from Creatinine
16.	NEWBERNE, Paul Massachusetts Inst. of Technology 5 R01 CA 26917-05	Dietary Fat in Colon Carcinogenesis
17.	PARIZA, Michael W. University of Wisconsin 5 R01 CA 29618-02	Structure and Origin of Mutagens in Fried Beef
18.	PAULING, Linus C. Linus Pauling Institute of Science 5 R01 CA 26541-02	Diet and Breast Cancer in Mice
19.	PAWLOWSKI, Norman E. Oregon State University 5 R01 CA 25766-04	Mechanisms for Biological Activity of Cyclopropenes
20.	REDDY, Bandaru American Health Foundation 5 R01 CA 36892-02	Macro and Micronutrient Interaction in Colon Cancer
21.	REDDY, Bandaru American Health Foundation 1 R01 CA 37663-01	Mechanisms of Dietary Fat Effects in Colon Cancer
22.	ROEBUCK, Bill D. Dartmouth College 5 R01 CA 26594-03	Modulation of Pancreatic Carcinogenesis by Diet
23.	ROGERS, Adrianne E. Massachusetts Inst. of Technology 3 R01 CA 25538-03S1	Dietary Fat, Prolactin and Mammary Cancer
24.	RUDOLPH, Frederick B. Rice University 5 R01 CA 14030-13	Regulation of Metabolism by Purine Interconversions
25.	SARKAR, Nurul H. Sloan-Kettering Inst. for Cancer Res. 5 R01 CA 25679-05	Effect of Diet on Murine Mammary Tumorigenesis

26.	SCANLAN, Richard A. Oregon State University 5 R01 CA 25002-12	Nitrosamines in Foods
27.	SELIVONCHICK, Daniel P. Oregon State University 5 R01 CA 30087-04	Membrane Protein Composition: Cyclopropanoid Fatty Acid
28.	SHINOZUKA, Hisashi University of Pittsburgh 5 R01 CA 26556-06	Diet Modification and Promotion of Liver Carcinogenesis
29.	SUMMERS, Jesse W. Institute for Cancer Research 5 R01 CA 16442-10	Regulation of Tumor Susceptibility
30.	THANASSI, John University of Vermont 5 R01 CA 35878-02	Vitamine B-6 Metabolism in Hepatomas
31.	THOMPSON, Henry J. University of New Hampshire 5 R01 CA 28109-05	Nutrition and Mammary Carcinogenesis
32.	TROLL, Walter New York University Medical Center 5 R01 CA 16060-13	Inhibition of Tumor Promotion by Protease Inhibitors
33.	VISEK, Willard J. University of Illinois 5 R01 CA 33796-02	Dietary Fat-Protein Interactions in Colon Cancer
34.	WEINDRUCH, Richard H. Univ. of California (Los Angeles) 5 R01 CA 26164-05	Dietary Restriction Cancer and Immune Functions
35.	WEISBURGER, John H. American Health Foundation 5 P01 CA 29602-03	Nutritional Carcinogenesis
36.	WELSCH, Clifford W. Michigan State University 1 R01 CA 37613-01	Caffeine and Experimental Mammary Gland Tumorigenesis

SUMMARY REPORT

MOLECULAR CARCINOGENESIS

The Molecular Carcinogenesis program area includes 260 grants with FY85 funding of approximately $26.44 million. There are no contracts in this program area. The grants consist of 241 R01 (Research Project) grants, 10 R23 (Young Investigator) grants, 5 P01 (Program Project) grants, 1 R43 (SBIR) grant and 2 R35 Outstanding Investigator grants. Research in the program area focuses on the characterization of carcinogen-macromolecule interactions (20 grants); changes in biological macromolecules and cell functions as a result of carcinogen or cocarcinogen exposure (28 grants); the identification of biochemical and molecular markers and properties of cells transformed by carcinogens (39 grants); the genetics and mechanisms of cell transformation (28 grants); the development of carcinogenicity/mutagenicity testing procedures (15 grants); the mechanisms of carcinogen-induced mutagenesis and genetic damage (18 grants); the identification and properties of tumor promoters and mechanisms of tumor promotion (52 grants); interspecies comparisons in carcinogenesis (11 grants); the genetics and regulation of enzymes associated with carcinogenesis induced by chemical and physical carcinogens (10 grants); development of analytical methodology for detecting chemical carcinogenesis in body fluids and environmental samples (4 grants); and the role of DNA repair in carcinogenesis (29 grants). Expanded descriptions of individual subject areas, along with examples of research accomplishments, are provided below.

Grants Activity Summary

Carcinogen-Macromolecule Interactions: The projects in this subject area focus on studies on the identification, quantitation and characterization of carcinogen-nucleic acid adducts. The interest in the identification and characterization of DNA adducts stems from the role alterations in DNA play in the initiation of carcinogenesis. Most of the carcinogens used in these studies are metabolized by cellular xenobiotic metabolizing enzymes to a variety of metabolites of which one or a few are reactive and bind to nucleic acids and/or proteins. The identification and quantitation of the binding species are generally determined by chromatographic and radioisotope techniques. The levels and persistence of specific DNA adducts are often related to the organ specificity of the carcinogen and indicate which of the adducts are biologically relevant. For many carcinogens such as the polycyclic aromatic hydrocarbons, alkyl nitrosamines, N-2-acetylaminofluorene, and aflatoxin B_1, the reactive metabolites and the identity of the various nucleoside adducts are known. The chemical nature and physical conformation of the adducts is thought to determine the biological effect of the adduct. For this reason, several investigators are focusing on the chemical and biophysical characterization of carcinogen-DNA adducts and on the resultant conformational changes the adducts may introduce into the DNA molecule. In many of the studies, defined polydeoxynucleotide sequences containing a modified base are synthesized for analysis. Several different techniques have been utilized for the characterization of carcinogen-nucleic acid adducts. These include high pressure liquid chromatography, absorption and fluorescence spectroscopy, nuclear magnetic resonance, optically detected magnetic resonance, linear and circular dichroism spectroscopy and x-ray crystallography. In addition, computer analysis of possible carcinogen-DNA adduct conformations has allowed the building of molecular models for the most likely conformations. Another determinant of the biological

effect of carcinogen DNA adducts is their potential site or sequence-specific interaction on the DNA molecule. The examination of this possibility for aromatic amine, polycyclic aromatic hydrocarbon and the metal carcinogens is the focus of several studies. The results of these studies give information as to the possible mechanisms by which a carcinogen may cause a mutation or other alteration in the DNA structure.

Alpha, beta-unsaturated carbonyl compounds, such as acrolein, crotonaldehyde and methylvinyl ketone are ubiquitous in the environment and occur in a wide range of natural and commercial products. These compounds have been shown to be mutagenic in bacteria in the absence of an activating system. Acrolein, which occurs in relatively high concentrations in cigarette smoke and is a primary metabolite of the chemotherapeutic drug, cyclophosphamide, was incubated with deoxyguanosine under physiological conditions in order to determine whether it reacts with DNA to give deoxyguanosine adducts. Three major products were isolated by high-performance liquid chromatography. These adducts were identified by their ultraviolet, mass, and nuclear magnetic resonance spectra, by the spectra of the corresponding guanine derivatives, and by chemical transformations. All products were shown to be cyclic 1,N^2-propanodeoxyguanosine derivatives (92). The number of other compounds that can form cyclic adducts with DNA is increasing rapidly. In addition to acrolein, alpha-acetoxy-N-nitrosopyrrolidine, 4-(carbethoxynitrosamino)-butanal, crotonaldehyde, glyoxal, 1,3-bis(2-chloroethyl)-1-nitrosourea, ethyl carbamate, chloroacetaldehyde, vinyl chloride, beta-propiolactone, glycidaldehyde, triose reductone, misonidazole and substituted malondialdehydes have been documented to form cyclic adducts. This class of nucleoside adducts, which is not yet well studied, may have general importance in mutagenesis and carcinogenesis. Because of this potential importance and the relative paucity of studies directed toward the biological activity of cyclic adducts, a program initiative is being developed to address this problem.

A determination of the role of individual as well as specific combinations of carcinogenic polycyclic aromatic hydrocarbon-DNA interaction products in the induction of neoplasia depends on the ability to separate and identify the individual DNA adducts formed in cells in culture and in vivo. Sephadex LH-20 and reverse-phase high performance liquid chromatography (HPLC) have been used to separate benzo(a)pyrene-deoxyribonucleoside adducts. However, neither method, has the ability to completely resolve all of the adducts formed from the reaction of syn- and anti-benzo(a)pyrene diol epoxide (BaPDE) with DNA. In this laboratory, a chromatographic procedure using boronic acid residues linked to a cellulose support, which was developed by Sawicki, Moshel and Dipple, was modified to allow the analysis of BaP-DNA adducts formed in cells in culture. In this system, adducts resulting from the reaction of anti-BaPDE contain cis-vicinal hydroxyl groups that complex with the boronic acid residues; adducts resulting from syn-BaPDE do not. A mixture of these adducts was shown to be completely resolved on a column of boronate-cellulose. Using a combined boronate chromatography-HPLC procedure, the separation of BaP-DNA adducts formed in mouse, rat and hamster embryo cells and a human hepatoma cell line was considerably improved. This facilitated the identification of the BaP metabolite(s) responsible for the formation of these adducts (7).

Chromium (VI) has been identified as a human carcinogen from epidemiological studies. Chromium (VI) compounds have been shown to be tumorigenic in animals and mutagenic in various bacterial and mammalian cell systems. Although chromium (III), which is produced upon reduction of chromium (VI), is the form ultimately

bound to cell DNA and protein, it produces no direct mutagenic and carcinogenic effects. An uptake-reduction model has been used to explain the carcinogenicity of chromium (VI) which can enter the cell using the sulfate transport system. Chromium (III), which forms octahedral complexes, is assumed to be impermeable to the cell membrane. The reduction of chromium (VI) to its active form has been shown to be associated with the cytochrome P-450 electron transport system. The binding of chromium to DNA and protein may occur during the production of intermediate, labile oxidation states and ultimately produce chromium (III) complexes bound to cellular macromolecules. The interaction of chromium (III) with poly(dG-dC) was shown to inhibit the B to Z transition and resulted in the condensation of the polymer at high chromium/nucleotide ratios. At low chromium/nucleotide ratios chromium (III) was shown to enhance the ability of ethanol to induce the B to Z transition of poly(dG-dC). From these results it appears that the biological effects of chromium (III) will depend on its concentration in the nucleus. Thus, chromium (III) may interfere with gene expression and gene regulation through its ability to alter the B to Z transition and cause DNA condensation in active chromatin (232).

There is epidemiological evidence for nickel as a human respiratory carcinogen as well as evidence that certain nickel compounds are carcinogens in animals. Both direct and indirect mechanisms have been suggested to explain the carcinogenicity of nickel compounds. There is evidence that nickel can act as a direct chemical mutagen in somatic cells, but not in bacterial cells. In previous studies nickel carbonate was shown to induce DNA lesions in vivo in rat tissues. The in vivo binding of nickel to chromatin, nucleic acids and nuclear proteins from rat kidney and liver was investigated in order to correlate the tissue and intracellular distribution of nickel in rats with the levels of nickel bound to cellular macromolecules and with nickel-induced DNA lesions. The level of nickel bound to whole chromatin from kidney was found to be higher than that from liver, and these levels could be correlated to the nuclear concentration of nickel. Much higher levels of nickel were found bound to the DNA-histone octamer complex and purified, deproteinized DNA from kidney as compared to liver. The results presented suggest that the formation of strong nickel-DNA and nickel-DNA-protein complexes in vivo may be related to the persistent DNA-protein cross-links observed in rat kidney following injection of nickel carbonate. Similar results were obtained using isolated nuclei. The results also suggest that DNA provides the inert coordination environment which stabilizes the nickel-DNA and nickel-DNA-protein complexes. Using the nuclei system, future studies will be aimed at identifying the nuclear proteins and DNA sequences involved in nickel-induced DNA-protein cross-links (232).

The metal carcinogen, chromium, has been shown to react with DNA and to cross-link nuclear proteins to DNA in isolated nuclei when it is converted to its active trivalent state. The biological significance of the chromium-mediated DNA-protein cross-linking is not known and the nature of the cross-linked proteins is not known. In this study, antibodies to nuclear nonhistone chromosomal protein fractions were used to attempt the characterization of the cross-linked proteins. The results showed that proteins of the nuclear envelope and nuclear matrix are selectively involved in this interaction. This could be due to their primary structure, their proximity to DNA, their accessibility to the cross-linker and what is thought to be more important, the chromatin conformation. Thus, it is speculated that chromium, by altering the relationship of the nuclear matrix to DNA, may affect and modify the regulation of DNA replication and RNA transcription and processing (100).

Changes in Cellular Macromolecules and in Cell Functions: The types of research activities in this subject area include studies on alterations in the composition and amounts of various proteins and small molecules, and changes in the pattern of DNA methylation in cells induced by carcinogens to the preneoplastic or neoplastic state. Biochemical and immunochemical methods have been used to isolate, identify and characterize nonhistone chromosomal proteins, phosphoproteins, and cytosolic proteins which are either altered or specifically appear in chemically induced hepatocarcinogenesis models. Neoplastic cells have been shown to manifest a variety of morphological and biochemical phenotypes different from their normal cell counterparts which are presumed to result from a substantial reprogramming of the cellular genome during neoplastic transformation. Not all of this reprogramming is thought to be due to direct alterations of the DNA genome. It has been hypothesized that non-DNA factors, so called "epigenetic" effects, play a role in the eventual appearance of neoplastically transformed cells. One possible manner in which the derepression and repression of genes could occur is by alterations in nuclear DNA-nuclear protein complexes. There is also much evidence showing that the state of DNA methylation regulates gene expression and also is involved in the control of cell differentiation. Thus, a greater understanding of the effects of carcinogens and other oncogenic agents on production of aberrant DNA methylation patterns during carcinogenesis is warranted. Several studies are being supported which seek to define the role of altered chromosomal protein-DNA complexes in carcinogenesis and to understand the role of DNA methylation in the control of gene expression and carcinogenesis. Some of the latter studies are focused on elucidating the properties and regulation of DNA methyltransferase, the enzyme responsible for the postreplication methylation of cytosine residues in DNA. Other studies are focused on the state of methylation of specific DNA sequences or genes as a result of carcinogen exposure. The biological effects of DNA hypomethylation, i.e., altered cell differentiation or induction of cell transformation, is being studied by using compounds such as 5-azacytidine, which are known to affect the transfer of methyl groups to DNA.

Studies in the laboratory of Lubomir S. Hnilica have focused on the nature and role of chromosome nonhistone protein-DNA complexes in cell differentiation and hepatocarcinogenesis. Polyvalent antisera, monoclonal antibodies, and immunotransfer methodology have been used to identify and characterize a group of chromosomal protein antigens which appear during azo dye hepatocarcinogenesis. In experiments designed to locate the placement of the antigens, it was shown that the majority of nuclear antigens were associated with high-speed DNA-containing pellets. Other experiments suggested the presence of protein-protein and/or protein-DNA complexes cross-linked with sulfhydryl linkages. Analysis of nuclear matrix preparations indicated that these antigens are components of the residual nuclear matrix, envelope, and/or associated structures and that they may undergo post-translational modifications. The results from this study suggest a possible role of the nuclear matrix and associated structures in neoplasia. The emerging picture from a considerable amount of recent evidence suggests that some physiological processes in the nucleus require a support structure at some point during these alteration events. The alteration of these structural components during carcinogenesis may thus represent a significant step during the transition to neoplasia (99).

Cells from malignant tumors characteristically exhibit changes in control of cellular function suggesting alterations in genetic modulation mechanisms. Since a variety of evidence supports the involvement of nonhistone chromosomal proteins in gene regulation, it was reasoned that changes in nonhistone chromosomal

proteins during chemical carcinogenesis might provide markers for, or might be involved in the genesis of malignancy. In this study two dimensional polyacrylamide gel electrophoresis and silver staining were used to analyze nonhistone chromosomal proteins at defined stages during liver carcinogenesis and in tumors induced by diverse acting carcinogens. The results obtained indicate that while N-2-acetylaminofluorene and diethylnitrosamine exposure results in a number of qualitative nonhistone chromosomal protein changes specific for the particular carcinogen, a total of only 10 changes, 7 inductions and 3 losses, were found to occur in common during hepatocarcinogenesis induced by these agents. The induction of 4 of these nonhistone chromosomal proteins during malignant conversion may be critical to the carcinogenesis process. Alternatively, they might serve as tumor markers since they were shown to appear first in a well-characterized premalignant state and to persist in resultant tumors (12).

Approximately 3 to 6% of cytosine residues in the DNA of all vertebrates is modified to 5-methylcytosine, which is predominately found in the dinucleoside sequence 5'-CpG. Substantial evidence has accumulated over the past several years that the methylation of cytosine residues in vertebrate DNA is implicated in the control of gene expression. The interrelationship between the effect of enzymatic DNA methylation on gene expression and neoplastic transformation is not clear as contradictory results on the levels of DNA methylation and methylase activity in tumors have been reported. As an example of this, one laboratory has studied the effect of DNA damaging agents (UV radiation, N-methyl-N-nitrosourea, and N-acetoxy-2-acetylaminofluorene) on genomic hypomethylation using transformed lymphoblasts and human diploid fibroblasts. None of the damaging agents were shown to produce a detectable change in methylation levels of newly replicated DNA, even at levels of damage that inhibited replication by 95%. The results of these experiments indicated that extensive demethylation is not a necessary consequence of DNA damage (142).

On the other hand, the genomic level of DNA cytosine methylation was shown to be significantly diminished in dividing BALB/3T3 A31 CL1-13 cells treated with several aromatic hydrocarbon carcinogens (benzo(a)pyrene and the syn- and anti-benzo(a)pyrene diol epoxides, and benzo(a)pyrene-4,5-epoxide). However, concentrations of some carcinogens which do not transform these cells were shown to also cause significant reductions in DNA cytosine methylation. The conclusion from this study was that the inhibition of DNA methylation may be an important step in the initiation of oncogenic transformation of BALB/3T3 cells, but that decreases in DNA 5-methylcytosine levels alone cannot account for the onset of the multistep process of cell transformation (115).

The mechanism by which benzo(a)pyrene diol epoxide (BPDE) modification of DNA influences the methylation reaction catalyzed by a highly purified DNA methytransferase from human placenta was investigated in one laboratory. The human DNA methyltranferase was shown to methylate both hemimethylated and 5-methylcytosine-free DNA substrates suggesting that this enzyme molecule exercises both maintenance and de novo activity. Modification of these methyl accepting polymers with anti-BPDE was shown to interfere with the methylation reaction, the level of inhibition being proportional to the degree of BPDE-modification. It was concluded that BPDE-DNA adducts affect both the maintenance and de novo DNA methyltransferase activity. BPDE-DNA adducts were shown to not interfere with the initial binding of the enzyme to DNA nor with the processive mode of action of the enzyme on the modified DNA template. From these experiments it was concluded that the modification of G-residues within or adjacent to CpG sequences is responsible for the impaired transmethylation reaction (227).

The above conclusion was also supported by work in another laboratory in which a rat liver DNA methyltransferase was used to catalyze the methylation reaction on anti-BPDE modified _M. luteus_ DNA and poly(dC-dG) templates. The presence of BPDE adducts was shown _to inhibit_ the methylation reaction on native DNA. Unlike the human enzyme, the rat liver enzyme was shown to not methylate the poly(dC-dG) polymer processively. Using circular dichroism measurements the possibility of a conformational shift from B- to Z-DNA was excluded as a cause of the inhibition of the methylation reaction. The data obtained suggested that an altered dG-base configuration and nearly irreversible enzyme binding to BPDE-modified DNA were responsible for the inhibition in methylation kinetics. Using aminofluorene- or acetylaminofluorene-modified poly(dC-dG) polymers in another experiment, similar conclusions were reached (138).

The studies cited above and other evidence has shown that chemical carcinogens, which chemically modify DNA, significantly decrease enzymatic methylation of DNA in vivo and in vitro. This has led to the hypothesis that demethylation may play a role in activation of the cancer cell phenotype. In contrast, one laboratory has recently shown that one chemical carcinogen, ethyl methanesulfonate inactivated specific gene expression apparently by promoting an increase in the enzymatic methylation of cultured cell DNA. Methyl methanesulfonate has also been reported to have this effect. Since it is known that ethyl methanesulfonate introduces ethyl groups primarily at the N-7 position of guanine and secondarily at the phosphate oxygens, it was proposed that ethyl methanesulfonate might promote enzymatic methylation by creating a "fraudulent" hemimethylated site by rotation of an ethyl group at guanine N-7 or the phosphodiester in close proximity to cytosine C-5 in CpG sequences in B-DNA. This ethylated site might then be recognized as hemimethylated by DNA methyltransferase and subsequently undergo methylation. A prediction of this model has been tested by using a synthetic polymer, poly(dC-dG)·(dC-dG), ethylated with increasing molar ratios of ethyl methanesulfonate. This increasingly modified polymer was shown to progressively stimulate its methylation in vitro by a partially purified rat DNA methyltransferase. Maximum stimulation was obtained when 2.7% of the guanine N-7s were modified. It was calculated that the enzyme had a relative affinity for the hemiethylated CpG, with N-7 ethylguanine mimicking a hemimethylated CpG site, 18-fold above unmodified CpG. With the ethylation of dioxyphosphate oxygen, the calculated affinity was up to 370-fold higher (109).

The exposure of cells to carcinogens directly affects DNA replication, RNA transcription and RNA transport from the nucleus to the cytoplasm. Several investigators are studying the mechanism of DNA replication following carcinogen-induced DNA damage. Other studies are focused on the characterization of the effects of carcinogen-modified DNA on RNA transcription and the mechanism of altered gene transcription and translation. A possible effect of carcinogen exposure is to alter the fidelity of DNA replication. The identification of cellular factors which control the fidelity of DNA synthesis such as altered DNA polymerases is being explored as well as the relationship between tumor progression and the fidelity of DNA replication.

Chloroacetaldehyde, a metabolite of vinyl chloride, has been shown to react with the exocyclic groups of adenosine and cytidine to first form an ethano ring structure which becomes dehydrated to 3,N^4-etheno C and 1,N^6-etheno A. The analogous guanine derivatives 3,N^2-etheno G and 1,N^2-etheno G are also formed. All exocyclic nucleoside derivatives except 1,N^2-etheno G have been found in DNA or deoxypolymers treated with chloroacetaldehyde in vitro, but only 3,N^4-etheno

C and 1,N^6-etheno A have been reported in nucleic acids of animals given vinyl chloride. There has been a question regarding the contribution of 1,N^6-etheno A and 3,N^4-etheno C to the carcinogenicity of vinyl chloride. Since biological effects are likely to occur by mutation, experiments have been designed to test whether the presence of the exocyclic compounds leads to errors during replication or transcription. In one study the mutational effectiveness of 1,N^6-etheno A was analyzed using transcription and replication by polymerases of high and low fidelity. Using E. coli DNA polymerase I (Pol I) and poly(dA) and poly(dA) containing 1,N^6-etheno dA, normal dTTP incorporation was found to be not significantly affected by the presence of 7% 1,N^6-etheno dA. dGTP misincorporation only occurred about once for every 500 1,N^6-etheno dA residues. The error-prone polymerase from avian myeloblastosis virus increased the error rate 5- to 20-fold to a maximum of 1 dG/25 1,N^6-etheno dA. No dCTP misincorporation was shown to occur with either enzyme. Transcription of the polymers by E. coli RNA polymerase revealed no errors when studied by nearest neighbor analysis. Increasing the amount of 1,N^6-etheno A in either synthesized or modified polymers caused a decrease in template activity without increasing the error rate. From these studies, it was suggested that 1,N^6-etheno dA generally does not prevent dT incorporation but behaves as a bulky lesion that is bypassed. The low mutagenic efficiency of 1,N^6-etheno A was contrasted to the high mutagenic efficiency of the lesion O^4-methyldeoxythymidine. The biological effects of some of the other exocyclic derivatives remain to be determined (64).

An unusually high error rate for the first nucleotide incorporated during DNA replication is predicted by mechanisms for fidelity that are mediated by a conformational change in DNA polymerase at each nucleotide addition step. This high error rate could be the reason for the use of oligoribonucleotides as primers for replication. Excision of RNA primers would then prevent the accumulation of mutations that would have resulted from error-prone synthesis at the start of DNA chains. To assess this prediction, an analysis of primer position effect on the fidelity of DNA polymerases alpha and beta was undertaken. Using synthetic oligodeoxynucleotides, the effect of primer position on single-base misinsertion frequencies at an amber site in phiX 174 DNA was measured. The results demonstrated a lack of position effect, an indication that processivity and the most direct "energy relay" proofreading mechanisms (which had been proposed to account for accuracy in the absence of exonucleolytic excision) are not important determinants in eukaryotic replication fidelity (198).

Markers and Properties of Transformed Cells: Research included in this subject area involves studies on the documentation of various growth and functional properties of initiated cells, preneoplastic cells and fully transformed cells, and the identification of biochemical and molecular markers for distinguishing these altered cell types from normal cells. The evidence obtained to date strengthens the supposition that the development of most cancers involves a multistep process in which cells progress from normal to initiated, preneoplastic, and premalignant stages to the end point of malignant neoplasia. In order to characterize cells at each stage, a detailed analysis and knowledge of the sequence of relevant biochemical and biological alterations associated with the development of chemically induced carcinogenesis is needed. To achieve this purpose, a variety of model systems, both in vivo in animals and in cells in culture, are being used. Of the animal model systems, the predominant one currently in use is the rat chemically induced hepatocarcinogenesis model. Although this model was established some time ago, the treatment regimens being employed have undergone a variety of changes depending on the purpose of the

experiment and on the endpoint desired. Chronic or intermittent exposure regimens have been used, along with initiation-promotion type regimens, in which various initiating carcinogens and promoting stimuli are used. The sequential appearance of foci of altered hepatocytes, nodules, and hepatocellular carcinomas can be observed and analyzed. There are other interesting model systems which are being established and analyzed by one or more laboratories. For example, a model of renal carcinogenesis in the rat is being established in which adenocarcinomas or mesenchymal tumors are selectively induced following a single dose of dimethylnitrosamine. Cell cultures representative of the renal tumor types are being established in order to correlate in vivo phenomena in renal carcinogenesis with events occurring in vitro.

Another interesting experimental system involves the establishment and sequential analysis of stages of oral carcinogenesis using hamster buccal pouch epithelium. The buccal pouch consists of a flat epithelium which has no glandular elements and normally lacks histochemical evidence of gamma glutamyltranspeptidase (GGT) activity. Whole-mounts of this epithelium can be prepared for analysis. Also, with this system it appears that it will be possible to relate the cells displaying altered growth in vitro to populations of presumptive initiation sites in vivo. This is not possible with other existing models.

Research relevant to respiratory carcinogenesis is being conducted using a rat tracheal implant system. The properties of carcinogen initiated cells can be studied in short-term organ culture where normal tissue interactions can be preserved. The cells can also be studied while growing in cell culture and also in vivo by allowing the cells to repopulate denuded trachea which are implanted into nude mice. Properties of normal and carcinogen-treated human respiratory epithelium can also be studied by using the denuded rat trachea implants in nude mice. These types of studies are being initiated and represent exciting new approaches to studying respiratory neoplasia and human respiratory neoplasia in particular. It should allow us to better extrapolate animal carcinogenesis results to their human counterpart. Research using other animal model systems, i.e., breast, colon, pancreas, bladder, and prostate, is being handled primarily by the Organ Systems Program of NCI, although some of these model systems are being used in projects supported by this program.

In addition to the utilization of animal systems, the in vitro transformation of cells in culture occupies the focus of several other research groups. The use of cell cultures which are derived from in vivo carcinogenic lesions allows investigators to analyze more easily properties of the cells in question. The ability to transform cells in culture allows for the study of mechanistic questions regarding chemically induced transformation. For some of this research, standard rodent fibroblast or epithelial cell lines have been used. With the increasing success in transforming human fibroblast and epithelial cells following the pioneering work of Kakunaga, Milo, and DiPaolo, several groups of investigators are increasingly turning to the use of human cell cultures in their research. This focus has been and will continue to be vigorously supported by NCI.

Upon transformation by chemicals, most cells acquire altered growth properties which allow them to proliferate under selective growth conditions. This can involve the ability to grow in soft agar (anchorage-independent growth), the loss of contact inhibition of growth, or the ability to grow in medium containing low calcium. Several biochemical and molecular markers have been used to identify transformed, preneoplastic and neoplastic cells. The histochemical expression of

GGT activity and the loss of histochemically determined glucose-6-phosphatase and ATPase activity are common markers used to identify carcinogen-altered liver cells and other epithelial cells. Other enzyme markers such as the presence of epoxide hydrolase, alkaline phosphatase isozymes and aldehyde dehydrogenase isozymes are being evaluated. Functional markers for liver cells being utilized currently include the production of albumin, alpha fetoprotein, transferrin, and fibrinogen. An increasing need is being seen for the development of genetic markers of neoplasia. The development of chromosomal abnormalities and aneuploidy in transformed cells are now being evaluated.

In a continuing study of oral carcinogenesis in the hamster buccal pouch epithelial system, the kinetics of induction and growth as well as the property of persistence of gamma-glutamyl transpeptidase (GGT)-stained cell populations induced by a 5-week regimen of biweekly topical applications of 7,12-dimethylbenz(a)anthracene (DMBA) was described. During the treatment regimen and at various times thereafter, GGT-positive foci were detected and quantitated in whole mounts of pouch epithelium. A comparison of GGT-positive foci at the completion of the DMBA regimen, and at 10 weeks thereafter, revealed no significant decrease in either the number or size of the foci. During the same 10-week post-treatment period, several dysplastic and occasional neoplastic lesions were observed in the pouch epithelium of DMBA-treated animals. Between the 15th and 34th weeks of the experiment, animals were sacrificed for histological and histochemical analyses. The data demonstrated that prolonging the biweekly DMBA treatment regimen from 3 to 5 weeks resulted in the formation of a greater number of GGT-positive foci, a proportion of which continue to express GGT activity for 10 or more weeks after DMBA treatment is stopped. A high incidence of dysplastic buccal pouch lesions was observed to develop as early as 7 weeks after the final DMBA application. The expression of GGT activity in early hyperplastic and dysplastic lesions and its persistence in several animals provide support for the investigators' working hypothesis that GGT-positive foci may represent sites of subsequent neoplastic development (207).

There is much evidence that many cell types exhibit an enhanced potential to grow in vitro shortly after carcinogen exposure. In in vivo/in vitro studies with a tracheal implant--cell culture system in which tracheas were preexposed to 7,12-dimethylbenz(a)anthracene (DMBA) for a short time--increased growth potential of carcinogen-altered tracheal epithelial cells was observed and identified by their ability to survive and grow in culture under stringent nutritional conditions in which unexposed cells never survived. The selective medium supporting the growth of carcinogen-altered cells was Waymouth MB 752/1 medium plus insulin, hydrocortisone and serum. Normal cells were shown to grow only when this medium was supplemented with amino acids, fatty acids, putrescine, and sodium pyruvate. It was recently determined that removal of just the pyruvate from the enriched medium brought about the death of normal cells, while carcinogen-altered cells continued to grow. In one set of experiments the loss of requirement for pyruvate by carcinogen-altered cells was utilized as a way of selecting out and quantitating the numbers of altered populations induced in tracheal explants exposed to DMBA for 2 weeks, 4 weeks or continuously. An explant outgrowth procedure was used to generate primary cultures from pieces of DMBA-exposed tracheas in order to expand epithelial cell populations with a minimum amount of disruption or loss of cells. Initial outgrowth of cells took place in enriched medium for 2 weeks to allow equal opportunity for proliferation of single or small groups of altered cells in the explant epithelium before pyruvate and insulin were removed to select out the altered cells and kill normal cells. Using this method, the heterogeneity

among the many cell populations generated from each trachea could also be analyzed. The study showed that the length of DMBA exposure had a marked effect on the number of selected (altered) cell populations induced in the tracheas. The numbers of selected cell populations per trachea obtained two months after 2 weeks, 4 weeks, and continuous exposure of tracheas were 1.8, 5.0 and 7.2, respectively. The numbers of selected cell populations was shown to not increase with time after exposure indicating that the maximal number of initiation sites was fixed at each exposure level. These, and earlier findings from this laboratory, have indicated that pyruvate is very important to the basic metabolism of normal tracheal epithelial cells, and that carcinogen exposure induces a fundamental change in this metabolism that gives a growth advantage to the carcinogen-altered cell early in the progression of neoplasia (158).

The relative abilities of populations of eukaryotic cells to grow in calcium-poor and calcium-rich media have been found in several studies to distinguish between tumorigenic and nontumorigenic populations of cells. Although the ability to grow in calcium-poor medium has been shown to be a characteristic feature of populations of transformed cells, the data available have not allowed the conclusion that tumorigenicity and the ability to grow in calcium-poor medium are closely coupled at the clonal or cellular level. In an attempt to clarify this problem, the relative tumorigenicity of a phenotypically heterogeneous population of hepatic epithelial cells transformed by multiple exposures to N-methyl-N'-nitro-N-nitrosoguanidine was compared with 14 clonal subpopulations that were isolated by their ability to form colonies in calcium-poor medium. The results obtained indicated that the selected clonal subpopulations of cells were phenotypically heterogeneous for gamma-glutamyl transpeptidase activity and anchorage-independent growth. They were also found to be less tumorigenic than the phenotypically heterogeneous parent cell line. From these observations, it was concluded that tumorigenicity and ability to form colonies in calcium-poor medium are independent phenotypic traits in populations of transformed hepatic epithelial cells and that these traits are not coupled closely in individual cells from which clones are derived (78).

Hepatocyte nodules have been consistently seen in every model of liver carcinogenesis well before the first appearance of hepatocellular cancer. Because of the central and presumably initial role of hepatocyte nodules in the development of liver cancer in the rat, it was considered to be of importance to understand much more about their biochemical properties. Initial studies were done to attempt to understand the biochemical basis for the resistant phenotype that allows for the genesis of nodules in the resistant hepatocyte model of liver carcinogenesis. The altered hepatocytes have the ability to proliferate to form nodules in the presence of a dose of a carcinogen that inhibits the proliferation of the majority of hepatocytes. These resistant hepatocytes exhibit a resistance to the necrogenic effects of CCL_4 and dimethylnitrosamine in vivo and a decrease in the metabolic conversion of dimethylnitrosamine and 2-acetylaminofluorene to DNA binding and mutagenic derivatives. As a possible basis for the resistant phenotype, components of the microsomal mixed function oxidase system, including cytochromes P-450 (phase I components) and the conjugating and detoxifying systems for xenobiotics and their metabolites were examined. These Phase II components included glutathione S-transferase and glutathione. For this study, nodules were induced by the resistant hepatocyte, choline-deficient, methionine-low diet, phenobarbital and orotic acid models of liver carcinogenesis. In addition, nodules generated by the resistant hepatocyte model were examined after transplantation to the spleen of syngeneic animals. Despite differences in the nature

of the models, the nodules showed an unusual degree of commonality in respect to a biochemical pattern. This consisted of decreased levels of cytochromes P-450, cytochrome b_5 and aminopyrine N-demethylase activity and increased levels of glutathione and gamma-glutamyltranspeptidase in whole homogenates and glutathione S-transferase activity in the cytosol. This similarity, appropriate to a resistant phenotype, is said to add additional support for the hypothesis that hepatocyte nodules may be a common step in liver carcinogenesis in several models (53).

Previous work in the laboratory of Ronald Lindahl has shown that a tumor-associated aldehyde dehydrogenase (ALDH) can be induced by a number of different carcinogens during rat hepatocarcinogenesis. This phenotype is characterized by an increase in total ALDH activity due to the appearance of several cytosolic isozymes undetectable in normal rat liver. Using at least three different protocols, the tumor-associated ALDH phenotype was shown to appear in later stages of hepatocarcinogenesis, concomittant with the appearance of overt tumors. These earlier studies were limited in that it was not possible to identify which liver cell populations were responsible for this change and at precisely what point during hepatocarcinogenesis this phenotype was first expressed. With the development of a histochemical procedure, it was demonstrated that the expression of the tumor-associated ALDH phenotype was limited to discrete focal preneoplastic cell populations, neoplastic nodules and hepatocellular carcinomas. The generality of the tumor-associated ALDH phenotype was further assessed using the resistant hepatocyte model. The hepatic ALDH phenotype was determined at intervals over 280 days by histochemical analysis, total ALDH activity assays and gel electrophoresis. Total activity assays and gel electrophoresis showed that no significant changes in ALDH activity occurred until day 70. However, changes in ALDH activity was demonstrated by histochemical analysis early in neoplastic development, being first detectable at day 28. The number of tumor-associated ALDH-positive foci was shown to increase until day 35 and then remain relatively constant for the remainder of the experiment. Gamma-glutamyl transpeptidase (GGT) activity of serial sections showed that early ALDH-positive lesions represented a small subpopulation (9%) of all GGT-positive foci. By day 168 about 80% of the persistent GGT-positive neoplastic nodules were also shown to be tumor-associated ALDH-positive, histochemically. Also, 96% of the hepatocellular carcinomas generated by this protocol were shown to possess significantly elevated levels of tumor-associated ALDH by histochemical analysis, total ALDH activity and gel electrophoresis. These results indicated that early-appearing ALDH-positive lesions may define one early subpopulation of all initiated cells that have a high probability of progressing to the ultimate neoplasm (145).

Genetics and Mechanisms of Cell Transformation: In the subject area of genetics and mechanisms of cell transformation are studies designed to test the somatic cell mutation hypothesis of cell transformation and to attempt the identification of those specific genes which are responsible or have an influence on cell transformation. There is a large body of data demonstrating a high correlation between the mutagenicity and carcinogenicity of various chemicals. This supports the hypothesis that somatic mutations are involved in the process leading to neoplasia. Somatic cell hybridization techniques have been used to study the types of mutations leading to the transformation of BHK cells by chemical carcinogens. The chemically induced transformants examined show the characteristics of a

a single-step, recessive mutation. Temperature-sensitive transformants which result primarily from base change mutations rather than frameshift mutations have also been isolated. Similar types of studies are being conducted to determine the number of complementation groups into which the various transformants isolated fall. This will allow a determination of the number of functional alterations which are needed to lead to the expression of transformation. In another study, the genetic mechanisms of lymphoma induction in mice by the dermal application of 3-methylcholanthrene (MCA) are being investigated. The major goal is to test whether resistance to MCA lymphomagenesis is controlled by a single gene and whether this same gene also confers resistance to radiation lymphomagenesis. This involves the use of various strains of mice in mouse genetic studies. Another approach to the above question involves the use of cells from individuals with a hereditary form of cutaneous malignant melanoma. This disease appears to be inherited as an autosomal dominant trait and most of these patients have preneoplastic melanocyte abnormalities, termed dysplastic nevus syndrome, which are correlated with a markedly increased risk of developing malignant melanoma. The biochemical basis for the increased sensitivity of non-malignant skin fibroblasts from these patients to 4NQO, a UV-mimetic carcinogen will be investigated.

In previous studies, it was shown that the permanent cell line BHK-21/cl 13 can be transformed by mutagenic carcinogens as the result of the induction of a recessive somatic mutation. This immortal cell line has been characterized as being one step removed from being tumorigenic. An alternative to the somatic mutation theory of carcinogenesis is that aberrant gene expression essential for carcinogenesis can also arise by epigenetic alterations in gene regulation analogous to those which mediate normal development and involve no substantial change in base sequences. This possibilty was tested using 5-azacytidine, a weakly or non-mutagenic agent which has been shown to cause undermethylation of cellular DNA and concomitant transcriptional activation of genes. BHK-21/cl 13 cells were efficiently transformed with 5 to 10 uM 5-azacytidine, doses which failed to produce mutants resistant to either ouabain or 6-thioguanine. When tested by cell fusion techniques, the transformants induced by 5-azacytidine fell into the same complementation group as those induced by highly mutagenic carcinogens. However, these transformants were phenotypically distinct in that they were unstable during prolonged passage and rarely displayed the temperature-dependent phenotypes that are common among BHK transformants induced by strongly mutagenic carcinogens. These results suggest that a cell can be induced by either genetic or epigenetic means to traverse the same single step in carcinogenesis (17).

Newer studies on the role of specific genes and gene products in chemically induced cell transformation have been initiated. The newly developed recombinant DNA, gene cloning, and DNA sequencing techniques have been employed in this research which has resulted in a veritable explosion of publications demonstrating the isolation and characterization of genes responsible for the transformation of cells to malignancy. To date, several different transforming genes have been isolated from different human tumor cells and their homology to various viral oncogenes has been established. Recently, it was shown that there is increased transcription of the cellular homologue of the transforming gene of Harvey sarcoma virus (c-Ha-*ras* gene) during the early stages of liver regeneration. Increased transcription of the c-Ha-*ras* gene was also observed during chemically induced hepatocarcinogenesis.

In further studies the transcription of six cellular oncogenes during the process of compensatory growth in rat liver after partial hepatectomy was examined. Polysomal poly(A)$^+$ RNA populations were obtained at various times after partial hepatectomy. The mRNAs corresponding to c-Ha-ras, c-Ki-ras and c-myc genes were shown to increase 2- to 10-fold with the highest increase for the c-Ki-ras gene transcript. Transcripts of c-abl and c-src were shown to be essentially unchanged and c-mos transcripts were virtually undetectable in either normal or regenerating rat liver. Changes in c-myc transcripts were shown to occur before DNA synthesis in liver regeneration after partial hepatectomy or CCl_4 injury. The elevations of c-myc and c-ras transcripts was shown to be sequential in that the highest levels of c-myc transcripts were detected 12 to 18 hrs. after partial hepatectomy, whereas the levels of c-Ha-ras and c-Ki-ras were maximal by 36 to 48 hrs. By 96 hrs. transcripts of all three activated oncogenes returned to their basal levels. The results suggest a role of c-myc in triggering the S phase or in inducing hepatocytes to enter the cell cycle. c-Ha-ras and c-Ki-ras genes may play a role concomitant with or subsequent to DNA synthesis (55). In a subsequent study the expression of the above six proto-oncogenes was examined during the course of hepatocarcinogenesis induced by a choline-deficient diet containing 0.1% ethionine. The abundance of c-Ki-ras, c-Ha-ras and c-myc transcripts in polysomal poly(A)$^+$ RNA from liver cells was shown to increase by 2 weeks after the start of the carcinogenic diet. c-Ki-ras and c-myc expression remained elevated during the 35 weeks of the diet, but c-Ha-ras transcripts were shown to only increase transiently. High levels of both c-Ki-ras and c-myc RNA were formed in a primary tumor sampled at 35 weeks after the carcinogenic diet was started. The abundance of c-src transcripts was shown to be unchanged throughout carcinogenesis and c-abl and c-mos transcripts could not be detected in either preneoplastic or neoplastic livers. Hepatocytes, oval cells and bile duct cells were isolated from normal and preneoplastic livers to determine which cell types within the liver contained the proto-oncogene transcripts. The results showed that proto-oncogenes are expressed differentially in these cell types during hepatocarcinogenesis and that the expression of c-Ki-ras and c-myc was high in oval cells throughout carcinogenesis. The results of these and other studies suggest that elevated c-myc expression is associated with hepatocarcinogenesis, whereas c-Ha-ras expression may be related to hepatocyte proliferation (55).

Several studies aimed at the identification of specific transforming genes or the activation of known previously identified oncogenes are currently ongoing or have just been initiated. Various chemically induced animal or cell model systems are being utilized which include rat hepatocarcinogenesis, mouse thymic lymphomas, rat nasal carcinoma, skin carcinoma, mouse bladder carcinoma and in vitro hamster fibroblast and epithelial cell transformation systems. For example, in one study the expression of the Ha-ras oncogene during the course of bladder carcinogenesis using the N-4-(5-nitro-2-furyl)-2-thiazolyl)formamide/Fischer rat and the butyl-4-hydroxybutyl-nitrosamine/C3H mouse bladder carcinogenesis models will be evaluated. The studies will involve the quantitation of the mRNA and gene to the sequencing of the gene, localization of the gene within cells and also its localization on the human chromosome. One other study involves the use of the mouse two-stage skin carcinogenesis model to determine whether altered expression of specific genes (several oncogenes, murine leukemia virus proviral sequences and long terminal repeat sequences) coincides with particular stages of carcinogenesis and/or tumor development. In fact, in one study in another laboratory, RNA sequences homologous to the long terminal repeat sequence of Moloney murine leukemia virus proviral DNA were found to be expressed in murine squamous cell carcinomas of the skin induced by chemical carcinogens. These RNA transcripts

were found to range in size from 8.2 to less than 2.4 kb with the size profile varying between individual tumors. These RNAs were not detected in the poly(A)$^+$ RNA fraction obtained from the epidermis of control mice or carcinogen induced skin papillomas. The results showed that chemical carcinogenesis in mouse skin is associated with constitutive expression of endogenous retrovirus-related sequences in the carcinomas as well as in certain apparently normal host tissues (227).

Another possible mechanism of cell transformation by chemicals could involve the induction of DNA sequence rearrangements, free radical intermediates or specific proteases. These changes could result in the altered cell growth and other properties characteristic of transformed cells. In one study the occurrence of DNA sequence rearrangements during hepatocarcinogenesis in rats and whether such rearrangements involve transforming genes is being examined using gene cloning, restriction endonuclease analysis and DNA transfection studies. In another project the role of DNA recombinational events, free radical intermediates, cell growth modification, patterns of cell differentiation and the induction of specific proteases is being examined in the mouse embryo C3H 10T 1/2 cell line and in a human diploid cell line transformed by chemicals and radiation.

Several studies have supported the role of free radicals in tumor promotion. In one study, experiments were designed to test whether radiation transformation in vitro, either with or without enhancement by 12-O-tetradecanoyl-phorbol-13-acetate (TPA), could be affected by agents which interact with free radicals. The effects of superoxide dismutase, catalase, Cu(II) (3,5-diisopropylsalicylate)$_2$(CuDIPS) and other copper compounds on radiation transformation in vitro using C3H 10T 1/2 cells was studied. High concentrations of superoxide dismutase in the medium when present only during irradiation was shown to enhance transformation. Catalase, inactivated superoxide dismutase, CuDIPS, cupric chloride and cuprous chloride were shown to inhibit the initiation phase of radiation transformation. Superoxide dismutase, catalase and CuDIPS were shown to not affect the expression phase of radiation transformation. Catalase significantly suppressed the TPA enhancement of transformation, while the suppression by superoxide dismutase was shown to be not statistically significant. The results obtained suggested that hydrogen peroxide, which is formed from superoxide anion radicals, may be of importance both in the initiation phase of in vitro transformation as well as in promotion in vitro by TPA (124).

Several hypolipidemic drugs, like clofibrate, and certain industrial plasticizers induce the proliferation of peroxisomes, enhance the activity of peroxisome-associated beta-oxidation of fatty acids and produce hepatocellular carcinomas in the livers of rodents. None of the carcinogenic peroxisome proliferators has been shown to be mutagenic in bacterial assays. They also display no capacity to covalently modify DNA either in vivo or in vitro. The presence of these properties has led to the hypothesis that the induction of hepatocellular tumors in hypolipidemic drug-treated rats is related to the persistent production of peroxisomes and the induction of peroxisomal oxidases that cause a sustained increase in intracellular hydrogen peroxide or other reduced oxygen species, which would then introduce mutagenic DNA damage. The ability of peroxisomes purified from the livers of normal and hypolipidemic drug-treated rats to induce DNA strand scission in vitro was investigated. Peroxisomes purified from the livers of hypolipidemic drug-treated rats produced a 30- to 70-fold increase in hydrogen peroxide generation when compared to controls. The levels of hydrogen peroxide generated correlated well with the induction of single-strand breaks in supercoiled SV40 DNA molecules that were included in the reconstituted peroxisome

incubations. The addition of excess catalase to the peroxisome incubations was shown to not prevent strand breaks, suggesting that other reduced oxygen species may be rapidly generated from hydrogen peroxide. The above results support a proposed mechanism in which hepatocellular genetic damage is introduced by the by-products of peroxisomal fatty acid beta-oxidation, an oxidative pathway that is substantially increased in hypolipidemic drug-treated livers (51,137).

Another major focus of projects in this subject area are studies designed to test the cell cycle specificity of the induction of cytotoxicity, mutagenesis, and neoplastic transformation by chemical carcinogens. Also, the quantitative relationship between the level, persistence, and species of carcinogen-nucleotide adducts and the concomitant cell transformation frequency are being determined. There is a substantial amount of information supporting the hypothesis of cell cycle specificity of carcinogenesis. It has been shown that in mouse embryo C3H 10T 1/2 cells, G_1 and S phase cells are susceptible to cytotoxicity and mutation, while only S phase cells (in synchronized cultures) are susceptible to neoplastic transformation by exposure to alkylating agents. In adult rat liver, the hepatocytes are generally resistant to carcinogenesis by a single exposure to agents capable of inducing cancer in other tissues. Hepatocyte susceptibility to carcinogenesis is increased by certain treatments which stimulate the proliferation of carcinogen damaged cells. Additional work is in progress to determine more specifically in rat liver the phase of the cell cycle which is most susceptible to the initiation effect of carcinogenic chemicals.

Development of Carcinogenicity Test Systems and Mechanisms of Mutagenesis: The development of carcinogenicity test systems subject area includes projects in which epithelial and fibroblast cell culture systems, specially constructed bacterial strains, erythroid cells, and a ^{32}P-labeling test are being used to monitor the effects of exposure to known and potential carcinogens. The end points being measured include cell transformation, mutagenesis, or the generation of DNA damage. Most tumor cells have the capability to proliferate in medium containing low calcium concentrations, while normal cells do not. Mouse epidermal cells can be subcultured in the absence of feeder layers in low calcium medium. In the presence of high calcium medium these cells cease to grow and terminally differentiate. Epidermal cells altered by chemical carcinogens, however, continue to grow in high calcium medium and do not terminally differentiate. This difference in growth response to high calcium is being used to select cells transformed by carcinogens. Work is in progress to isolate and characterize cloned epidermal cell lines for use in a tester system, to identify and resolve the sources of variability in this system and to identify additional markers of transformation of epidermal cells.

In another laboratory epidermal cells from skin tumor sensitive (SENCAR) mice are being used to develop a reliable and quantitative in vitro transformation system. In this system the epidermal cells are grown on a mouse fibroblast feeder layer in medium containing standard calcium levels. Under these conditions the epidermal cells can grow, be subcultured and terminally differentiate (stratify, form keratin, and cornify) in a manner analogous to normal skin. It was felt that this should allow studies of epidermal carcinogenesis and differentiation to be conducted under more normal conditions. The SENCAR mouse skin tumorigenesis system has been used extensively for the testing of chemical compounds for their carcinogenic activity using an initiation-promotion protocol. Investigations on the critical biochemical events in initiation and promotion have been conducted using this animal model. The development of an in vitro transformation system

should facilitate investigations into mechanistic questions involving initiation and promotion as well as allowing the detection of carcinogens and promoters.

The bacterial mutagenesis systems currently in use have been much less successful in predicting the carcinogenicity of metal compounds than they have for predicting the activity of organic compounds. In one project a bacterial short-term test system with a genetic endpoint of broad specificity for detecting carcinogenic metal compounds is to be developed. The investigator has developed a microtitre assay using a number of E. coli B strains which allow the analysis of toxicity, lambda prophage induction and mutagenicity of metal compounds simultaneously. The system will be developed further to increase its sensitivity and to broaden the genetic endpoints detectable. These studies will be extended to the Chinese hamster V79 cell system, which have the known capability to phagocytize insoluble metal compounds. This will allow the study of particulate metal compounds which do not enter bacteria.

The basis for development of a different bacterial test system for carcinogens comes from a theoretical paper published by Dr. Martin Pall (Proc. Natl. Acad. Sci. USA 78: 2465-2468, 1981) in which he describes a mechanism by which carcinogenesis might occur. The mechanism proposed is that initiation involves a mutation which produces a tandem duplication of certain genes (a proto-oncogene is suggested) and that promotion involves the further amplification of the same genes by unequal crossing over and sister chromatid exchange. When sufficient gene product is produced, the cell will then become transformed. In the proposed work certain predictions of the theory will be tested. The main prediction to be tested using derivatives of the bacterium, Salmonella typhimurium, will be that carcinogenic chemicals can cause tandem duplications more efficiently than noncarcinogenic chemicals can. The results obtained to date have confirmed this prediction in that tandem duplications of the histidine operon were induced by a number of direct-acting chemical carcinogens but not by toxic agents. Some problems with the current procedure for selecting for tandem duplications have been noted. Further work to make the system more specific and to investigate the mechanism of tandem duplication induction is in progress. It is felt that the work can lead to the understanding of some molecular events in carcinogenesis and to a potentially valuable carcinogen test system.

The proposition that gene rearrangements or gene amplification may be more significant in carcinogenesis than classical point mutations is the rationale for a project to construct a mammalian cell line derived from Chinese hamster V79 cells, which will have deletion mutations in the cell's thymidine kinase (TK) genes and a single copy of the herpes simplex virus TK gene. With this system the principal investigator is expected to be able to determine whether a given carcinogen or tumor promoter produces TK+ to TK- mutations and if so, whether these involve base pair substitution, frameshift mutations, or gene rearrangements or gene amplification. Gene amplification or gene rearrangements will be determined by using restriction endonuclease and Southern blot analysis.

There is considerable interest in developing methods that will allow investigators to determine whether people have been exposed to harmful levels of chemical carcinogens. Of the laboratories that are developing such methods, two of them are focusing on measuring the level of base substitution mutations produced in erythrocytes. Monoclonal antibodies are being produced which can recognize mutant hemoglobin and spectrin molecules. These will be labeled by conjugation with

fluorescein. Erythrocytes are labeled with the fluorescently conjugated antibodies specific for the variant proteins. The mutant cells are then enumerated using high-speed fluorescence-activated cell sorting and automated scanning microscopy. The method has the capability of detecting one abnormal cell in 10^7 cells and thus the frequency of background and mutagen/carcinogen-induced somatic mutations can be determined. In another laboratory, the goal of the proposed work is to develop a quantitative in vitro assay for measuring the increase in frequency of thioguanine-resistant T lymphocytes in humans exposed to environmental mutagens. Because practical methods to measure frequency of mutants induced in vivo have not been available, indirect measurements, such as determining the frequency of chromosome damage, sister chromatid exchanges or the induction of tumors are often used. These methods have limitations in applicability due to the relatively low number of cells examined in chromosome-chromatid studies and the long latent period required for cancer. The recent isolation of the T-cell growth factor, interleukin 2, now makes it possible to grow human T-cells isolated from peripheral blood. The proposed series of experiments, if successful, may ultimately allow the evaluation of mutagen dose in cases of human exposure. In a similar system using normal human T-cells and T-cell leukemia/lymphoma, the proposed studies will determine the efficacy and feasibility of combining the electrophoretic multiple locus mutation system and T-cell cloning procedures to analyze the role and impact of somatic cell mutagenesis in vivo and in vitro.

The goal of one study is to investigate factors associated with susceptibility to mammary cancer and to develop methods to identify environmental chemicals that may induce mammary cancer. Cultured rat mammary cells are being characterized for use in a direct clonal specific locus mutagenesis assay system or as activating cells in a cell-mediated mutagenesis assay system. A parallel human mammary gland in vitro system is also being developed. The susceptibility of rats to polycyclic aromatic hydrocarbon (PAH)-induced mammary carcinomas has been shown to be modified by both genetic background and physiological state. The physiological state of pregnancy has been shown to confer significant resistance to PAH-induced mammary tumorigenesis. Variations in carcinogen metabolism have been suggested as a mechanism to alter susceptibility to chemically-induced tumors in both experimental animals and humans. Results obtained in earlier studies suggested that the ability to activate PAHs is probably not the controlling factor in genetically controlled susceptibility to chemically induced breast cancer. In this study, the abilities of primary mammary cells from pregnant and virgin rat cultured on collagen-coated plates to metabolize PAHs and convert these carcinogens to mutagenic derivatives was tested. Mammary epithelial cells from pregnant rats were found to produce half the levels of mutagenic 7,12-dimethylbenz(a)anthracene (DMBA) metabolites formed by cells from virgin rats. These cells also showed consistently lower levels of metabolism of DMBA and benzo(a)pyrene (BaP) than cells from virgin rats. High performance liquid chromatography analysis of BaP metabolism by these cell populations indicated no significant qualitative differences in the extracellular and intracellular metabolites formed. The results obtained suggested that, unlike genetically controlled susceptibility, physiologically modulated susceptibility may be associated at least in part with an altered ability to activate PAHs in the mammary gland (73).

A method of detecting the in vivo formation of carcinogen-DNA adducts that does not require the use of radiolabelled test compounds has recently been developed. The procedure involves the incorporation of (^{32}P)orthophosphate into carcinogen

nucleotide adducts obtained by digesting DNA from experimental animals that had been treated with the test compound. The labelled adducts are then resolved by thin layer chromatography and the radioactivity detected by autoradiography and scintillation counting. A modification of the technique was applied to the study of the adducts formed in liver DNA of adult CD-1 mice given a single dose of a series of alkenylbenzenes, plus allylbenzene and isosafrole. The long-term persistence of safrole-DNA adducts in mouse liver and the influence of the sulfotransferase inhibitor, pentachlorophenol, on safrole-DNA adduct formation was also investigated. The known hepatocarcinogens, safrole, estragole and methyleugenol was shown to exhibit the strongest binding to mouse liver DNA, when analyzed 24 hrs. after administration of the test compounds. Several related compounds (allylbenzene, anethole, myristicin, parsley apiol, dill apiol and elemicin), which have not been shown thus far to be carcinogenic in rodent bioassays, were found bound to mouse liver DNA at 3-to 200-fold lower levels. No binding was detected for eugenol. Low binding to mouse liver DNA was also observed for the weak hepatocarcinogen, isosafrole. The main adducts observed appeared to be guanine derivatives. Adducts formed by reaction of 1'-acetoxy-safrole with mouse liver DNA in vitro were determined to be chromatographically identical to safrole-DNA adducts found in vivo. The pretreatment of mice with pentachlorophenol was shown to inhibit the binding of safrole to mouse liver DNA. This provides further evidence that the metabolic activation of the allylbenzenes proceeds by the formation of 1'-hydroxy derivatives as proximate carcinogens and 1'-sulfooxy derivatives as ultimate carcinogens (181).

In addition to the development of mutagenicity test systems are projects which seek to understand how mutations and DNA or chromosome damage are generated by carcinogenic chemicals. Specifically synthesized oligonucleotides of defined base sequence are being used to examine the molecular mechanism of frameshift mutagenesis. The base sequence specificities of the interactions of frameshift mutagens with oligonucleotides are being studied and correlated to their mutagenic activity in Ames tester strains having known base composition in the frameshift site. Studies are also being supported for the study of the mechanism and genetic control of frameshift mutagenesis in yeast. The recently sequenced His4 gene system with existing (+1 G/C) and new (-1) frameshifts are to be used to construct tester strains. DNA sequencing and recombinant DNA technology have been used in these studies. The types of studies to be undertaken include an assessment of the effect of transcription on mutation frequency, the effect of having an origin of replication in close proximity, an analysis of gene duplication, and a study of the potential role of the nuclear envelope in mutagenesis.

Mutagenesis by aflatoxin B_1 is being investigated at the molecular level in a prokaryotic experimental system involving E. coli, phage ΦX174, the plasmid pBR322 and/or various M13 phage cloning vectors. It has been demonstrated that the activated form of aflatoxin B_1 causes the covalent modification primarily of guanine residues at the N-7 positions, leading to alkali-labile sites in DNA. DNA sequence analysis was used to identify alkali-labile sites induced by aflatoxin B_1 and to determine the frequency of alkali-labile aflatoxin B_1 modifications at particular sites on a DNA fragment of known sequence. Previous results have shown that, in general, all guanine residues in single-strand DNA are equally subject to aflatoxin B_1 modification and that the DNA sequence does play a role in the site of binding of aflatoxin B_1. The effect of aflatoxin B_1-induced chemical modification on the template function of single stranded DNA in vitro was analyzed using the single-strand phage M13 DNA. The data showed that aflatoxin B_1 induces specific replication blocks one nucleotide 3' to the sites of occurrence of guanine residues on template DNA and that the aflatoxin B_1-induced replication

blocks occurred predominantly at sequences capable of participating in intrastrand base pairing. Within the intrastrand base-paired regions strong sequence context effects were observed in accordance with previously described specificity "rules" that apply to the reaction of aflatoxin B_1 with guanine residues in double-stranded DNA. A plausible mechanism for the observed sequence specificity has been suggested to involve a precovalent association in the major groove of B-DNA between aflatoxin B_1 and particular sequences in double-stranded DNA (105).

Newer studies in this area have focused on the use of specific genes which will either be cloned into plasmids or are present in cellular DNA as targets for the mutagenic action of various chemical carcinogens. The c-Ha-ras oncogene cloned into a plasmid, the lac gene introduced into M13 phage DNA and the dihydrofolate reductase gene in Chinese hamster ovary cells are being used as target genes to assess the mutagenic action of chemicals such as benzo(a)pyrene diol epoxide, N-acetoxy-2-acetylaminofluorene, and other aromatic amines. DNA sequence techniques and effects of lesions on DNA synthesis will be used to determine the mechanisms of mutagenesis.

DNA damage also leads to aberrations at the higher organizational level of the chromosome. Projects are being supported whose goals are to understand the molecular mechanisms which lead to the formation of chromosome aberrations and to investigate the biological significance of the sister chromatid exchange assay. Standard cytogenetic techniques as well as cell fusion techniques to form prematurely condensed chromosomes, DNA elution techniques, and techniques required to get drugs and enzymes into cells are being utilized to study chromosome damage by various chemical agents. Controversy still exists over the molecular basis for chromosome aberration formation. Many types of lesions might be the primary lesions involved in aberrations, including base damage and double-strand breaks. One of the difficulties in elucidating the molecular basis of chromosome aberration formation is that most clastogens induce a variety of types of DNA lesions, and it is difficult to decipher which lesions are crucial. An attempt was made in one study to determine the role of double-strand breaks in chromosome aberration formation. The experimental strategy employed was to generate single-strand gaps in quiescent normal human fibroblasts by inhibition of excision repair of nitrogen mustard lesions with cytosine arabinoside and hydroxyurea and then to convert these lesions into double-strand breaks by treatment of cells with a single-strand-specific neurospora endonuclease. The cells are then fused with mitotic cells and the aberration frequency is directly determined in the G1 prematurely condensed chromosomes. The results show that this treatment significantly increased the aberration frequency above that of the repair-inhibited but not endonuclease-treated control. This confirms the hypothesis that double-strand breaks are important in the formation of chromosome aberrations (96).

A combination of classical somatic cell genetic and recombinant DNA technologies are being used to study the effects of chromosome rearrangements on gene expression and mutation or deletion. Also, a cell line that is heterozygous for four different linked genetic or cytogenetic markers will be utilized to assay mitotic recombination, gene inactivation, chromosomal rearrangements, deletions, or chromosome segregation events leading to the expression of APRT-recessive mutant phenotypes in mutagen-treated or untreated cell cultures. This system will be used to determine whether extramutational events are induced by known tumor initiators or promoters. In the Chinese hamster ovary (CHO) cells used, recessive mutant phenotypes have been obtained at high frequencies. This has been generally attributed to the extensive functional hemizygosity of the CHO genome. Although

CHO cells have an approximately diploid DNA content, more than half of the chromosomes in the CHO karyotype reflect translocations and other rearrangements that appear to have occurred during the evolution of this cell line. In order to gain further insight into the nature of hemizygosity in CHO cells and the mechanisms by which it has arisen, the mapping and linkage relationships of as many hemizygous drug resistance or enzyme marker loci in CHO cells as possible is being attempted. About 25% of the 43 enzyme loci for which electrophoretic mobility shift mutants have been obtained have been shown to be hemizygous. The chromosomal location of many of these hemizygous gene loci remain to be determined. The segregation of methylglyoxalbisguanyl hydrazone (MBG) resistance, a previously unmapped drug resistance marker, with other enzyme markers has been examined using intraspecific CHO cell hybrids. MBG is a potent inhibitor of polyamine synthesis. On the basis of segregation data, the MBG locus was determined to be linked to the hemizygous isocitrate dehydrogenase 2 locus on chromosome Z3. Nine of the 10 autosomal hemizygous gene loci that have been mapped to date in CHO cells have been shown to be clustered on 3 chromosomes, with 5 markers on chromosome 2, 2 on chromosome 8, and now 2 on chromosome Z3. With the mapping of MBG to the Z3 chromosome, selectable drug resistance markers are now available on 8 different CHO chromosomes (2).

A new protocol for inducing mutations in mammalian cells in culture by exposure to the thymidine analog, 5-bromodeoxyuridine was recently established. This protocol, called "DNA-dependent" mutagenesis, involves the incorporation of 5-bromodeoxyuridine into DNA under nonmutagenic conditions and the subsequent replication of the 5-bromouracil-containing DNA under mutagenic conditions, but with no 5-bromodeoxyuridine present in the culture medium. The mutagenic conditions were induced by allowing DNA replication in the presence of high thymidine concentrations which generates high intracellular levels of dTTP and dGTP and causes nucleotide pool imbalance. The mutagenesis induced by this protocol was found to correlate with the level of 5-bromouracil substituted for thymidine in DNA in contrast to a "pool dependent" protocol in which mutagenesis is dependent upon the 5-bromodeoxyuridine triphosphate pool levels (38).

In three other studies, methods for the analysis of mutations induced in human or other mammalian cells at the DNA sequence level are being developed. The approaches used depend on the use of recombinant DNA shuttle vectors composed of the simian virus (SV40) early region, sequences derived from the bacterial plasmid pBR322, and the herpes simplex virus thymidine kinase gene. A shuttle vector containing, in addition, the _E. coli lac_ I gene has also been used. The studies seek to determine the types of DNA sequence changes induced by chemical carcinogens, to characterize host processes that determine the frequency or types of mutations induced by specificity in mammalian mutagenesis.

In one study a series of shuttle vectors introduced into a variety of mammalian cells (COS7 and CV-1 simian cells, NIH 3T3, 3T6, L, and C127 mouse cells, and human 293 and HeLa cells) in order to establish the generality of an earlier observation of high mutagenic frequency. DNA transfection has become a tool of pivotal importance in studying the molecular biology of mammalian cells, and has facilitated studies of the expression of manipulated genes and the identification of sequences of oncogenic potential. Several laboratories are now seeking to use DNA transfection to study mutagenesis in mammalian cells. The transfection of papovavirus-based shuttle vectors carrying the bacterial _lac_ I gene into cells was shown to yield a mutation frequency in the range of 1%. This frequency is approximately 4 orders of magnitude higher than the spontaneous mutation frequency

in either mammalian or bacterial cells. The mutations have been shown to be predominantly base substitutions and deletions and also include insertions from the mammalian genome. Time course experiments appear to indicate that mutagenesis occurs soon after arrival of the transfected DNA into the nucleus. Replication of the vector was shown to not be required for mutagenesis. The general picture which emerges is of a sizable mutagenic effect upon transfection which is probably ubiquitous among mammalian cells. The investigators speculate that the absence of chromosomal proteins on the transfected DNA may subject this DNA to the actions of degradative enzymes which are normally limited by the presence of such nuclear proteins (28). A similar high spontaneous mutation frequency was observed in another laboratory in which a recombinant shuttle vector, pSV_2gpt, which carries the *E. coli* gene for xanthine-guanine phosphoribosyltransferase (*gpt*), was introduced into Chinese hamster V79 cells. A 1% frequency of Gpt^- plasmids was observed and most of the mutant plasmids were characterized as having rearrangements in the region containing the *gpt* gene (38).

Properties and Mechanisms of Tumor Promotion: Research in this subject area involves projects designed to analyze the various cellular, biochemical and molecular activities and pleiotropic effects induced in cells upon exposure to tumor promoters. The phorbol ester tumor promoters are by far the most widely used compound in these studies. They have been shown to exert their effects by binding to specific receptors on the cell surface membrane. A number of grants support studies on the characterization of the phorbol ester receptor protein. The results of phorbol ester binding include alterations in membrane phospholipid metabolism, membrane structure and function, alterations in the transport of small molecules, the activation of macromolecular synthesis, the induction or inhibition of terminal cell differentiation by normal or neoplastic cells and the mimicry of the transformed phenotype by normal cells and the enhancement of transformation by chemicals and oncogenic viruses. Two of the studies are focused on the perturbation of ion fluxes by the tumor promoter, 12-O-tetradecanoyl-phorbol-13-acetate (TPA). Since the action of TPA may be mediated by the phosphorylation of proteins and lipids, several studies are focused on the purification and characterization of protein kinase C, a calcium and phospholipid-dependent protein kinase whose activity is stimulated by TPA. A characterization of the proteins phosphorylated by this enzyme is included in some of these studies. In some of the studies, the effect of TPA on cell differentiation is being characterized. The role of free radicals in promotion, either the active oxygen species generated by TPA in cells or the hydroperoxy fatty acids generated during the induction of the arachadonic acid cascade by TPA and other first and second stage promoters, is the focus of three of the studies. The activation of the expression of certain genes is thought to occur during neoplastic progression of cells. The possible activation of oncogene sequences and other viral and cellular gene sequences by TPA and other promoters is the focus of at least five studies.

Since humans are not normally exposed to phorbol ester tumor promoters, it was deemed necessary in 1981 to stimulate more research on agents more relevant to human exposure which might function as tumor promoters. To accomplish this a Request for Grant Applications (RFA) was issued inviting grant applications from interested investigators for both basic and applied studies that would seek to provide insight and approaches to an understanding of the role of tumor promoters, hormones and other cofactors in human cancer causation. The studies were to be focused on one or more of five different categories: (1) the development of nonphorbol tumor promotion or cocarcinogenesis models in experimental animals using the breast, colon, lung, prostate, stomach, urinary bladder, and/or uterus

organ systems; (2) the development of nonphorbol tumor promotion or cocarcinogenesis models in human and/or nonhuman cell and/or organ culture systems; (3) the study of the possible tumor promotion role of hormones and substances such as bile acids, saturated/unsaturated dietary fat, alcohol, salt or oxygen-free radicals; (4) the identification and elucidation of the mechanisms of action of non-phorbol tumor promoters and/or cocarcinogens; and (5) interdisciplinary studies involving epidemiologists and experimentalists to test hypotheses concerning tumor promotion generated by either.

In FY82 12 grants were funded from applications submitted in response to this RFA; ten were approved for three years of funding and two for four years. The role of dietary fat on DMBA-induced mammary carcinogenesis in rats or mice was the focus of two of the studies. The cocarcinogenic action of ethanol with nitrosamines in the oral cavity, esophagus and larynx of rats, mice and hamsters is the focus of one study. The rates of metabolic activation of nitrosamines in the target organ and cell cultures was to be measured. In a mouse lung tumor model, the mode of action of butylated hydroxytoluene (BHT) as a tumor promoter is being examined. The metabolism of BHT, the activation of cyclic GMP-and calcium-dependent protein kinase, the effect of glucocorticoids on urethane tumorigenesis and tumor promotion and the effect of BHT on glucocorticoid receptor localization is being studied. Using a heterotopically transplanted rat bladder system one laboratory is investigating the promoting effect of urine components on bladder carcinogenesis induced by N-methyl-N-nitrosourea and N-butyl-N-(3-carboxypropyl)-nitrosamine. The hypothesis that asbestos and selected non-asbestos minerals act as tumor promoters in carcinogenesis of the respiratory tract is being studied using a hamster trachea model. The determination of whether EBV-related oncogenic mechanisms in in vitro virus-cell interaction models involves promotion is to be made in one laboratory. The hypothesis given is that a viral mediated increase in an intracellular protein that blocks the viral lytic cycle and interferes with cell differentiation leads to uncontrolled cell proliferation and the ultimate selection of neoplastic cells. A study of the tumor-promoting activity of a number of anthracene derivatives such as chrysarobin and its synthetic analogs and homologs, which are related to anthralin, is being conducted using the 7,12-dimethylbenz(a)anthracene skin tumor model system. Two in vitro model systems for testing for tumor promoters are being developed. One model system uses hepatocytes or liver cells from carcinogen-treated rats which are then promoted in culture using selected compounds. The other model system uses various rodent and human cells to test the hypothesis that the induction of mutations at the HGPRT locus by promoters in hypermutable cells is a common property of cancer cells. In one study on the mechanism of action of promoters, the ability of promoters to stimulate gene amplification to methotrexate resistance is being studied. Finally, one of the studies involves a biochemical epidemiology project in which sex hormone levels in breast and prostatic cancer will be studied.

In FY85 competitive renewals for 7 of the 10 projects originally funded for 3 years have been submitted and reviewed. Only one grant application to support studies on the mechanism of action of a nonphorbol ester tumor promoter, chysarobin, will definitely be renewed in this fiscal year. It is hoped that another grant application to support studies on the potential role of oxygen free radicals in asbestos induced bronchogenic carcinoma will become fundable by the end of the fiscal year. Some interesting results have been obtained from studies supported through the RFA. These obtained will be described in greater detail below. It is also evident that this RFA has stimulated more studies on nonphorbol tumor promoters of relevance to humans. Some recently funded projects seek to

study the activity of compounds such as n-alkanes, cyclosporin A, endogenous hepatic growth modulators, hormones and dietary L-tryptophan as tumor promoters. In addition, epithelial cell and organ culture systems from human endometrium and human prostate are being developed to study the process of tumor promotion by a variety of agents such as hormones and TPA.

There is evidence that the use of oral contraceptive steroids by humans is associated with an increased incidence in benign liver neoplasms. In experimental studies in animals, results have been obtained which suggest that synthetic estrogen treatment following carcinogen exposure can enhance hepatic neoplasia. One study was designed to compare the enhancing activities of mestranol and ethinyl estradiol, to analyze dose-response effects and to determine the incidence of hepatocellular carcinomas. In addition, the effect of the addition of beta-methasone, a growth inhibitory corticosteroid for liver, was also determined. In diethylnitrosamine-initiated female Sprague-Dawley rats, ethinyl estradiol and mestranol were shown to cause 3.5- and 4.4-fold increases, respectively, at 9 months in the number of gamma-glutamyl transpeptidase (GGT) lesions per liver and an increased incidence of hepatocellular carcinomas, while estradiol had no enhancing effect. A significant decrease in GGT lesion number, but not carcinoma incidence, was demonstrated by the addition of beta-methasone to the mestranol-containing diet as compared to mestranol alone. The results confirm and extend previous studies and the results of others which indicate that synthetic estrogens can act as promoters of hepatocarcinogenesis (239).

Cyclosporine is a powerful new immunosuppressant in all species tested including humans, and is being used on patients undergoing organ transplants. A complication of cyclosporine therapy, as with conventional immunosuppressive agents, is the occurrence of lymphoproliferative disorders and lymphoreticular neoplasms. Recently, it was observed that dietary administration of cyclosporine to rats, in a dose comparable to clinical use, induced proliferative alterations of lymphoid tissues in the intestine and lymph nodes. Sequential morphological changes occurring in the lymphoid organs of rats on a cyclosporine diet for 8 to 10 weeks were examined and changes observed were correlated with changes in electrophoretic patterns of serum proteins. The lymphoid lesions induced in rats were shown to bear striking similarities to the post transplant lymphoproliferative lesions in patients receiving cyclosporine and in patients with the acquired immune deficiency syndrome (AIDS) (200).

Using the mouse skin two-stage carcinogenesis model and subsequent models that define stages of promotion, retinoic acid and its analogs have been well documented as inhibitors of 12-O-tetradecanoylphorbol-13-acetate (TPA) tumor promotion. However, there are several reports that retinoic acid may enhance the carcinogenic process. Retinoic acid has been shown to act as either a weak first stage promoter or a weak complete promoter in the initiation-promotion protocol for skin carcinogenesis in the SENCAR mouse. The retinoid analog RO-10-9359 was shown to lack this tumor promoting activity. In one of the properties examined, retinoic acid but not RO-10-9359 induced an immediate chemiluminescence response in human polymorphonuclear cells. Both retinoids, however, were shown to inhibit a TPA-induced response. Since the chemiluminescence response is believed to be due to oxygen free radical generation, the data suggest that the ability of retinoic acid, but not RO-10-9359, to promote tumors may be due to initial oxidative reactions at the cell membrane (59).

The development of a fully malignant tumor is thought to involve complex interactions between environmental and endogenous factors and often proceeds through several discernable stages (initiation, promotion, progression). During the multistage carcinogenic process, it was hypothesized that tumor promoters might act synergistically with cellular oncogenes, since promoters have been shown to induce mimicry of transformation, modulate differentiation and enhance the transformation of cells previously exposed to chemical carcinogens, radiation or certain DNA viruses. In examining possible synergistic interactions between tumor promoters and a cloned oncogene, C3H 10T 1/2 cells were used as recipients due to their more uniform fibroblastic morphology and a lower saturation density than NIH 3T3 cells. In this study, the tumor promoters, 12-O-tetradecanoylphorbol-13-acetate and teleocidin, were shown to markedly enhance the transformation of C3H 10T 1/2 mouse fibroblasts when these cells were transfected with the cloned human bladder cancer c-Ha-ras oncogene. Transfection studies with the drug resistance marker gpt and time course studies indicated that the observed enhancement was not due simply to an effect on the process of DNA transfection. The results obtained suggested that during multistage carcinogenesis, tumor promoters may complement the function of activated cellular oncogenes (228).

Tumor promoters have been shown to cause a variety of effects in cultured cells, at least some of which are thought to result from activations of the Ca^{2+}-phospholipid-stimulated protein kinase C. One action of tumor promoters has been shown to be the modulation of the binding and phosphorylation of the epidermal growth factor (EGF) receptor in A431 cells. In order to determine if these compounds act on the EGF receptor by substituting for the endogenous activator of protein kinase C, diacylglycerol, the effects of the potent tumor promoter 12-O-tetradecanoylphorbol-13-acetate (TPA) was compared with those of a synthetic diacylglycerol analog, 1-oleyl 2-acetyl diglycerol (OADG). The treatment of A431 cells with either TPA or OADG resulted in a shift in the subcellular distribution of protein kinase C activity from a predominantly cytosolic location to a membrane-associated state, which was correlated with changes in binding and phosphorylation of the EGF receptor. From the results obtained, it appeared that tumor promoters can exert their effects on EGF receptors by substituting for diacylglycerol, presumably by activating protein kinase C. It is also suggested that endogenously produced diacylglycerol may have a role in normal growth regulatory pathways (190).

The activation of protein kinase C was thought to require added Ca^{2+}. However, in one study using a bovine brain preparation of calcium- and phospholipid-dependent protein kinase C, it was observed that the maximal stimulation (greater than 10-fold) of kinase activity by 12-O-tetradecahoylphorbol-13-acetate (TPA) occurred in the presence of phospholipid but in the absence of added Ca^{2+}. In effect, mM concentrations of TPA were shown to substitute for nM concentrations of added Ca^{2+}, the two agents not acting synergistically. Similar activation in the absence of Ca^{2+} was observed with biologically active analogs of TPA and with the chemically unrelated tumor promoters teleocidin and aplysiatoxin, but not with biologically inactive compounds. The results obtained were considered to be consistent with a stereochemical model in which the hydrophilic domains of certain diterpenes, teleocidin and aplysiatoxin, interact specifically with the protein kinase C apoenzyme, while their hydrophobic domains interact with phospholipid, thus forming an enzymatically active ternary complex. Diacylglycerol, a putative endogenous activatior of protein kinase C, somewhat resembles the 12 and 13 positions of TPA. However, the chemical structures of mezerein, teleocidin and

aplysiatoxin do not suggest simple analogies to diacylglycerol. It is thus suggested that it may be worthwhile to search for more complex endogenous lipids that would activate protein kinase C with greater potency than the simple diacylglycerols that have been examined thus far (228).

In one of the studies supported under the "Promotion" RFA, it was previously shown that the amphibole types of asbestos, crocidolite and amosite, had the ability to induce squamous metaplasia in hamster tracheal epithelium. Also, chemical carcinogens such as polycyclic aromatic hydrocarbons (PAHs) have been shown to cause hyperplastic and metaplastic changes in tracheobronchial epitheluim both in vivo and in vitro. Tracheal organ cultures were used to assess a hypothesized synergistic action of asbestos and benzo(a)pyrene (BaP), a major PAH in cigarette smoke, to increase the extent of squamous metaplasia and proliferation of various epithelial cells in the respiratory tract. When exposed to either crocidolite asbestos or BaP, the epithelium of hamster tracheal explants exhibited an insignificant amount of squamous metaplasia, an atypical lesion, in comparison to amounts observed in untreated tissue. DNA synthesis in epithelial cells, as measured by (^{3}H) thymidine incorporation was shown to be likewise unchanged. However, when BaP and asbestos are added in combination, the extent of squamous metaplasia and numbers of basal and suprabasal cells were shown to be increased substantially. The results suggest an important mechanism of cocarcinogenesis involving chemical and physical carcinogens and support epidemiologic observations documenting an increased risk of bronchogenic carcinoma in asbestos workers who smoke (168).

In another "Promotion" RFA project, the role of dietary fat in the development of mammary cancers induced by dimethylbenz(a)anthracene (DMBA) was being studied. High fat diets were shown to enhance the development of tumors. In a current study, the objective was to test the hypothesis that there is a minimal requirement of essential fatty acid, linoleic acid being the major essential fatty acid, for mammary tumorigenesis and to delineate what this requirement is. Female Sprague-Dawley rats, induced for mammary tumorigenesis by DMBA, were fed diets containing different levels of linoleic acid but containing the same total dietary fat level, 20% by weight. This was achieved by combining varying amounts of coconut oil and corn oil. Mammary tumorigenesis was shown to be very sensitive to linoleic acid intake and increased proportionately in the range of 0.5% to 4.4% of dietary linoleic acid. From a regression analysis, the level of linoleic acid required to elicit the maximal tumorigenic response was estimated to be around 4%. The differences in tumor yield could not be correlated with changes in prostaglandin E concentration in the mammary fat pads of normal animals maintained on similar diets. This suggested that linoleic acid may act by some other mechanism than the stimulation of prostaglandin synthesis to stimulate mammary tumorigenesis (107).

Interspecies Comparisons in Carcinogenesis: In the subject area of interspecies comparisons in carcinogenesis are studies undertaken as a result of a specific initiative from the Branch to fill a perceived need to develop scientifically sound methodology for the extrapolation of carcinogenesis data derived from studies on experimental animals to humans. The initiative was designed to encourage studies that would be supportive of the Environmental Protection Agency in the area of risk assessment. In 1980, a RFA was issued inviting grant applicatons from interested investigators for both basic and applied studies designed to provide insight and approaches to an understanding of similarities and

differences in the response to chemical carcinogens between experimental animals and humans. The proposed studies were to emphasize the use of accessible human cells, tissues, body fluids and excreta and to focus on quantitative relationships related to the carcinogenesis process.

In FY 81, 16 grants were funded from applications submitted in response to this RFA; 15 were approved for three years and one for a five year period. Fourteen of the grants supported studies with either human cells only or with human and other rodent or monkey cells. One grant supported comparative studies in mice and rats only and another used hamsters, mice and rats. Fourteen of the grants supported studies on the comparative metabolism of a variety of chemical carcinogens such as polycyclic aromatic hydrocarbons, aromatic amines and nitrosamines. The development of human hepatocyte, pancreas, esophagus and bladder in vitro cell transformation systems was the additional goal in four of the projects. The development of direct or cell-mediated mutagenesis or genetic damage assay systems was also the focus in three of the studies. The primary goal in two of the funded studies was to develop techniques to measure mutant proteins in peripheral blood lymphocytes or in red blood cells from individuals who had been exposed to potential mutagens or carcinogens either as the result of various clinical procedures or from environmental or occupational exposure to chemicals.

By the end of FY 85 only 4 of the 16 original projects will still be active. Five investigators have chosen to not submit competing renewal applications. Of the 11 projects for which competing renewal applications have been received, only 3 have received funding as of the end of FY 85. These 3 were all renewed in FY 84. The goal of one of these projects is to provide an understanding of the mechanisms responsible for the metabolic activation, organ specificity, and species and strain specificity of the carcinogen methylazoxymethanol. Another project involves the continued development of equivalent rat and human mammary gland in vitro systems for ultimate interspecies comparisons of interactions of chemical carcinogens with these cells. The third project involves the further development and characterization of cell culture and transplantation systems of human hepatocytes obtained from normal and cirrhotic liver. The goal of the one RFA response approved for 5 years until FY 86 is to purify and characterize the several individual forms of cytochrome P-450, epoxide hydrolase and NADP-cytochrome P-450 reductase from human liver.

While it is not yet clear what the overall impact of the funded studies will have on the ability to extrapolate animal carcinogenicity data to humans, it is clear that the initiative on interspecies comparisons in carcinogenesis has stimulated additional studies using cells from human tissues, which will increase our knowledge on the metabolism and processing of carcinogens by those tissues and on the biological and molecular characteristics of the cells transformed by chemical carcinogens. In addition, many more investigators utilize more than one species or strain of animal in their proposed studies. Different animal species have been shown to be either sensitive or resistant to the action of various xenobiotics. Thus, use of different species can give insights into the mechanism of action of carcinogens in carcinogenesis. Subsequent to the RFA, 3 other projects have been added to this category of research.

Pancreatic carcinoma represents a major clinical problem affecting more than 22,000 individuals in the United States each year. The five-year survival rate for this disease is only 3%. A lack of adequate knowledge of the causes and factors influencing its biological behavior together with difficulties in the early detection of pancreatic carcinoma has contributed to its poor prognosis.

The development of a model system to study this disease in vitro would thus be of importance. A model system using adult human pancreas in explant culture was developed in the laboratory of Ismail Parsa. This permitted some studies on the effect of alkylating carcinogens on the various cell types. However, it was reported that acinar cells underwent degeneration and necrosis within 6 to 12 weeks in this model. In order to study the effects of chemical carcinogens on both acinar and ductal cells, an organ culture model of fetal human pancreas was developed. This resulted in explants that could be cultured in a chemically defined medium for up to 12 months in the presence or absence of methylnitrosourea (MNU). Differentiation of the exocrine pancreas was demonstrated in vivo. Treatment with MNU caused changes in acinar cells and proliferation of cells bearing duct cell markers. Enchanced foci of proliferation developed within 3 months and carcinoma within 5 months of treatment. Cells derived from 4- to 5- month MNU-treated explants were shown to be tumorigenic in nude mice (175).

It had been previously established that there are interindividual variations in the metabolism of foreign compounds, such as therapeutic drugs by humans. Genetic polymorphisms contribute in large part to the large interindividual differences observed. Debrisoquine 4-hydroxylase activity is considered to be a prototype for genetic polymorphism in oxidative drug metabolism in humans; about 10% of caucasian populations exhibit a poor metabolizer phenotype, and the clearence of at least 14 other drugs has been shown to be deficient in patients exhibiting this phenotype. Prior to conducting studies on the human debrisoquine 4-hydroxylase enzyme, a minor cytochrome P-450 was purified (to homogeneity) from Sprague-Dawley rat liver using debrisoquine 4 hydroxylase activity as an assay. This cytochrome P-450 (designated $P\text{-}450_{UT\text{-}H}$) was characterized by gel electrophoresis, peptide mapping and immunochemical analysis. Antibodies prepared to the rat cytochrome $P\text{-}450_{UT\text{-}H}$ were found to inhibit the oxidation of debrisoquine and sparteine, encainide and propranolol, three other drugs suggested to be associated with this phenotype, in human liver microsomes. The oxidation of seven other cytochrome P-450 substrates was not inhibited by the antibodies. The antibody was shown to recognize a single polypeptide of MW 51,000 following polyacrylamide gel electrophoresis and immunochemical staining of human liver microsomes. When liver microsomes from 44 organ donors was examined, the intensity of the stained band was found to be significantly correlated with debrisoquine 4-hydroxylase activity. By the immunoprecipitation of in vitro translation products of total liver RNA, it was calculated that the level of translatable mRNA coding for the debrisoquine-hydroxylating cytochrome P-450 is about an order of magnitude less in human liver than in rat liver. The availability of these antibodies will provide a biochemical basis for further basic and clinical studies on the role of a particular cytochrome P-450 polymorphism in humans (83).

Genetics and Regulation of Enzymes Associated with Carcinogenesis: Research projects in this subject area are focused on the use of somatic cell genetic and molecular biological approaches to study the regulation of the levels of carcinogen metabolizing enzymes. In 9 of the projects, the development and use of cloned cDNA probes to cytochrome P-450 or epoxide hydrolase genes form part of the proposed studies. These cloned cDNA probes are used to examine the levels of expression of the mRNAs for the genes in response to the modulation of enzyme activity by various chemicals. The genomic organization of the genes can also be examined. In two of the projects, mutants or variant cells, altered in their ability to induce the carcinogen metabolizing enzymes, are being isolated and characterized. Using the inbred hamster model the genetic variation in arylhydroxamic acid acyltransferase, sulfotransferase, N-acetyltransferase, and N-deacetylase enzymes which are involved in the metabolism of aromatic amines is

being studied. The characterization, localization and regulation of the enzyme arylsulfotransferase IV is the focus of one other study.

Aryl hydrocarbon hydroxylase (AHH), a cytochrome P-450 containing enzyme complex, and epoxide hydrolase are enzymes which are involved in the activation of polycyclic aromatic hydrocarbons (PAHs) to an ultimate carcinogenic derivative. Certain P-450 and non-cytochrome P-450 dependent activities can be induced in cells by PAHs and compounds such as 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD). The induction of these various enzymes has been shown to be mediated by a cytosolic receptor protein (the Ah receptor) which binds PAHs and then translocates to the nucleus, where it is believed to enhance transcription of the relevant genes. Both dominant and recessive AHH-deficient mutants of the mouse hepatoma line, Hepa-1, have been isolated in the laboratory of Oliver Hankinson. Using somatic cell genetic techniques, the recessive mutants have been shown to belong to three complementation groups, the group B mutants possessing greatly reduced amounts of Ah receptor and the group C mutants being characterized as unable to translocate the PAH-receptor complex into the nucleus. The primary defects in the group A recessive mutants and in the dominant mutants have not yet been identified. The purpose of the present study was to measure cytochrome P_1-450 mRNA levels and AHH activity in wild type Hepa-lclc7 cells and in AHH-deficient mutants derived from this cell line. TCDD was shown to induce both parameters about 50-fold in Hepa-lclc7 cells, but group B and C gene mutants and dominant mutants had nondetectable or much reduced cytochrome P_1-450 mRNA levels and AHH activities. Somatic cell hybirds between wild type cells and the dominant mutants were shown to also be deficient in the above parameters. The interpretation suggested by these results is that the dominant mutants are expressing a trans-acting repressor of cytochrome P_1-450 transcription. The properties of gene A mutants were shown to be heterogeneous. Some completely lacked the cytochrome P_1-450 mRNA; others were inducible for it; while still others (subgroup IV) had high levels even when grown in the absence of TCDD. These results suggested strongly that gene A is the structural gene for cytochrome P_1-450. This conclusion was also supported by studies in which a gene A mutant Hepa-1 cell line was treated with calcium phosphate precipitates of DNA from Hepa-1, the rat cell line H4IIEC3, or an A-/human hybrid in which the A^- mutation is complemented by the corresponding human gene. AHH^+ transfectants were isolated by the previously developed selection procedure using benzo(g,h,i,)perylene plus near UV light. Transfectants were found to be unstable during culture, rapidly losing AHH activity. Using the Southern blot technique, with a mouse cytochrome P_1-450 cDNA probe, all rat DNA-derived transfectants were shown to contain the rat cytochrome P_1-450 gene. In subclones of one of the transfectants, AHH activity and the rat P_1-450 gene segregated together. The results with the dominant mutants suggest that the cytochrome P_1-450 gene can be subject to both negative and positive control. Further studies on the group A mutants may show that these mutants provide a potentially powerful system for studying the mechanism of regulation of the cytochrome P_1-450 gene (88).

In other studies using the same Hepa-lclc7 mouse hepatoma cells, the laboratory of James P. Whitlock has used a cDNA probe which is specific for a PAH-inducible form of cytochrome P-450, P_1-450, to analyze the accumulation of enzyme-specific mRNA in wild-type Hepa-lclc7 cells and in variant cells defective in AHH induction. A population of variant cells with a markedly increased ability to metabolize benzo-(a)pyrene was also isolated by using a fluorescence-activated cell sorter. These variant cells exhibited increased AHH activity and increased responsiveness to TCDD induction. Cell fusion experiments demonstrated that the variant phenotype was co-dominant with respect to wild type. Filter hybridization experiments

indicated that the increased accumulation of cytochrome P_1-450-specific mRNA was responsible for the overproduction of AHH activity. By measuring RNA synthesis in isolated nuclei, the variants were shown to have an increased rate of transcription of the cytochrome P_1-450 gene in response to TCDD. The variant cells were shown to contain no detectable TCDD receptor alterations nor cytochrome P_1-450 gene amplification. Filter hybridization analyses of restriction endonuclease-digested DNA showed that the variant cytochrome P_1-450 gene is relatively undermethylated, compared to the wild type gene. From these results, it was concluded that the variant cells contain an altered cis-acting genomic element(s) which regulates the expression of the cytochrome P_1-450 gene. Further studies in this laboratory have shown that the transcription of the cytochrome P_1-450 gene in wild type Hepa-1 cells and in variant cells containing functional TCDD-receptor complexes can be superinduced by the inhibition of protein synthesis. The isolation and analysis of a DNA fragment containing sequences flanking the 5' end of the cytochrome P_1-450 gene showed that this DNA fragment contained a cis-acting control element with at least three functional domains: a putative promoter, an inhibitory domain upstream from the promoter that blocks its function, and a TCDD-responsive domain still further (1265 to 1535 base pairs) upstream of the promoter. From these findings, along with results from earlier studies, the investigators have suggested that the transcription of the cytochrome P_1-450 gene is under both positive and negative control by at least two trans-acting regulatory factors. One appears to be a TCDD-responsive element which presumably interacts with the TCDD-receptor complex; the other is suggested to be an inhibitory element, which interacts with a cycloheximide-sensitive repressor (234).

The cytochrome P-450s, though functionally related in catalyzing the oxidation of endogenous and foreign compounds, comprise a family of enzymes that differ from one another in primary structures, substrate specificities, antigenic characteristics and spectral properties as well as in their induction response to various xenobiotics. However, research directed at both the protein and nucleic acid sequence levels has clearly shown the existence of other closely related isozymes with extensive homology to previously known forms. The basis for this microheterogeneity is thought to be primarily genetic, with each slightly variant cytochrome P-450 being coded by a separate gene; or an allelic form of a gene, which is itself a member of a gene family. In one laboratory, the genetic localization of two cytochrome P-450 gene families, the NADPH-cytochrome P-450 oxidoreductase gene, and the epoxide hydratase gene was studied. A cDNA probe to a major rat phenobarbital-induced cytochrome P-450 mRNA (P-450 PB) and a cDNA probe to a major rat pregnenolone-16 alpha-carbonitrile-induced species (P-450 PCN) was used to detect cytochrome P-450 sequences. These gene families were shown to be genetically divergent from each other and showed no cross-hybridization. Using mouse x Chinese hamster somatic cell hybrids (EBS cell lines), all distinguishable P-450 PCN sequences were found to map to chromosome 6, whereas all P-450 PB sequences were located on chromosome 7. The data presented support the proposition that the region of the Coh locus on chromosome 7 is the site of the cytochrome P-450 PB gene family. NADPH-cytochrome P-450 oxidoreductase which appears to be encoded in many vertebrate species by a single gene, was found to be located on chromsome 6. The data presented showed that the Eph-1 locus on chromosome 1 is the site of at least one microsomal epoxide hydratase gene (164). Other studies in the laboratory of Daniel W. Nebert at NIH have demonstrated the localization of cytochromes P_1-450 and P_3-450 genes to mouse chromosome 9. These cytochrome P-450 species are induced by exposure to polycyclic aromatic hydrocarbons.

MOLECULAR CARCINOGENESIS

GRANTS ACTIVE DURING FY85

Investigator/Institution/Grant Number	Title
1. ACS, George Mount Sinai School of Medicine 5 R01 CA 16890-09	Studies on Chemotherapeutic Deoxyribonucleosides
2. ADAIR, Gerald M. University of Texas Sys. Can. Ctr. 2 R01 CA 28711-04	Expression of Genetic Variation in Cultured Cells
3. ALBERT, Roy E. New York University 5 P01 CA 26724-04	Inhalation Carcinogenesis of Environmental Agents
4. ALBERTINI, Richard J. Univ. of Vermont and St. Agri. College 5 R01 CA 30688-03	Direct Mutagenicity Testing in Man
5. ASHENDEL, Curtis L. Purdue University West Lafayette 5 R01 CA 36262-02	Interactions of Tumor Promoters with Receptors
6. AVADHANI, Narayan G. University of Pennsylvania 5 R01 CA 22762-08	Cellular and Molecular Targets of Chemical Carcinogenesis
7. BAIRD, William M. Purdue University West Lafayette 5 P01 CA 30234-03	Molecular Mechanisms of Carcinogen-DNA Interactions
8. BAKER, Donald G. Univ. of Virginia (Charlottesville) 2 R01 CA 25890-03	Influence of Hyperthermia on X-ray Carcinogenesis
9. BAXTER, C. Stuart University of Cincinnati 1 R01 CA 34446-01A1	In Vivo Immunotoxicology of Tumor-Promoting Agents
10. BAXTER, C. Stuart University of Cincinnati 1 R01 CA 36183-01A1	Tumor Promotion by Environmental N Alkanes
11. BECKER, Frederick F. University of Texas Sys. Can. Ctr. 5 R01 CA 20657-09	Phenotypic Analysis of Chemical Carcinogenesis
12. BECKER, Frederick F. University of Texas Sys. Can. Ctr. 5 R01 CA 28263-05	Chromosomal Proteins During Chemical Carcinogenesis

	Investigator / Institution / Grant	Project Title
13.	BENFIELD, John R. City of Hope National Med. Ctr. 2 R01 CA 29373-04	Model of Bronchogenic Lung Cancer
14.	BERNHARD, William A. University of Rochester 5 R01 CA 32546-09	Effects of Ionizing Radiation on Nucleic Acids
15.	BERRY, David L. U.S. Agricultural Research Service 5 R01 CA 28968-03	Mode of Action of Phorbol Esters in Epidermal Cells
16.	BIRT, Diane F. University of Nebraska Med. Ctr. 5 R01 CA 33368-02	Urinary Bladder Cancer Promotion by Dietary L-Tryptophan
17.	BOUCK, Noel P. Northwestern University 2 R01 CA 27306-07	Genetic Analysis of Malignant Transformation
18.	BOWDEN, George T. University of Arizona 5 R01 CA 26972-06	Postreplication Repair in Cultured Mammalian Cells
19.	BOX, Harold C. Roswell Park Memorial Institute 5 R01 CA 29425-05	Molecular Studies of Carcinogenesis and Mutagenesis
20.	BRANSCOMB, Elbert W. Univ. of California (Berkeley) 5 R01 CA 31714-03	Monitoring In Vivo Somatic Mutations In Animals and Man
21.	BRASITUS, Thomas A. Michael Reese Hospital and Med. Ctr. 5 R01 CA 36745-02	Colonic Epithelial Cell Plasma Membranes
22.	BRESNICK, Edward University of Nebraska Med. Ctr. 5 R01 CA 35994-02	Epoxide Hydrolase in Hyperplastic and Neoplastic Livers
23.	BRESNICK, Edward University of Nebraska Med. Ctr. 5 R01 CA 36106-03	Polycylic Hydrocarbon Metabolism and Carcinogenesis
24.	BRESNICK, Edward University of Nebraska Med. Ctr. 5 R01 CA 36679-03	DNA Repair After Polycyclic Hydrocarbon Administration
25.	BROYDE, Suse B. New York University 5 R01 CA 28038-05	Carcinogen-DNA Adducts: Linkage Site and Conformation
26.	BUTEL, Janet S. Baylor College of Medicine 5 R01 CA 33369-03	Tumor Promotion and Murine Mammary Cancer

	Investigator / Institution / Grant Number	Title
27.	BYUS, Craig V. Univ. of California (Riverside) 1 R01 CA 35807-01A1	Mechanism of Tumor Promoter Action
28.	CALOS, Michele P. Stanford University 5 R01 CA 33056-03	Mutation in Human Cells at the DNA Sequence Level
29.	CARTER, Timothy H. St. John's University 1 R01 CA 37761-01	Regulation of Transcription by a Tumor Promoter
30.	CHANG, Ching-Jer Purdue University West Lafayette 5 R01 CA 35904-02	Chemical Carcinogens and DNA Interactions in Tissue Culture
31.	CHRISTMAN, Judith K. Mount Sinai School of Medicine 5 R01 CA 25985-06	Response of Phagocytic Leukocytes to Tumor Promoters
32.	CLARKSON, Judith M. University of Texas Sys. Can. Ctr. 5 R01 CA 19281-09	Cell-Cycle Related DNA Repair Mechanisms
33.	CORDEIRO-STONE, Marila Univ. of North Carolina Chapel Hill 5 R01 CA 35657-02	Effects of BPDE on DNA Replication in Human Fibroblasts
34.	COSTA, Max Univ. of Texas Hlth. Sci. Ctr. (Houston) 2 R01 CA 29581-04	Mechanism of Metal Carcinogenesis
35.	CRAIGHEAD, John E. Univ. of Vermont and St. Agri. College 5 R01 CA 36993-02	Experimental Asbestos-Induced Mesothelioma
36.	CURPHY, Thomas J. Dartmouth College 5 R01 CA 30650-03	Pancreas & Liver Carcinogen Metabolism in Three Species
37.	DANIELSON, Keith G. Baylor College of Medicine 1 R23 CA 38650-01	Transformation of Mammary Epithelial Cells In Vitro
38.	DAVIDSON, Richard L. University of Illinois Med. Ctr. 5 R01 CA 31781-05	Mechanisms of Chemical Mutagenesis in Mammalian Cells
39.	DI MAYORCA, Giampiero Univ. of Medicine and Dentistry of NJ 5 R01 CA 25013-07	Molecular Mechanism of Chemical Carcinogenesis
40.	DIAMOND, Leila Wistar Inst. of Anatomy and Biology 5 R01 CA 23413-07	Tumor Promoter and Cell Differentiation

	Investigator / Institution / Grant	Title
41.	DIAMOND, Leila Wistar Inst. of Anatomy and Biology 5 R01 CA 30446-03	Hydrocarbon Activation by Cells
42.	DIAMOND, Leila Wistar Inst. of Anatomy and Biology 1 R01 CA 37168-01A1	Chemical Transformation, Neoplastic Progression and Oncogenes
43.	DIEBOLD, Gerald J. Brown University 5 R01 CA 29912-03	Optoacoustic Detection of Carcinogens
44.	DIGIOVANNI, John University of Texas Sys. Can. Ctr. 5 R01 CA 37111-02	Mechanism of Mouse Skin Tumor Promotion by Chrysarobin
45.	DIGIOVANNI, John University of Texas Sys. Can. Ctr. 1 R01 CA 38871-01	Genetics of Susceptibility to Skin tumor Promotion
46.	DRINKWATER, Norman R. Univ. of Wisconsin (Madison) 5 R01 CA 37166-02	Molecular Analysis of Carcinogen Induced Mutations
47.	DUFFEL, Michael W. Pennsylvania Hospital (Philadelphia) 1 R01 CA 38683-01	Aryl Sulfotransferase in Drug and Xenobiotic Metabolism
48.	DUKER, Nahum Temple University 5 R01 CA 24103-05	Molecular Pathology of Carcinogenic DNA Damage
49.	ESSIGMANN, Ellen M. SISA Pharmaceutical Labs., Inc. 1 R43 CA 36651-01A1	A Rapid In Vivo Prescreen for Hepatocarcinogens
50.	ESTENSEN, Richard Univ. of Minnesota (Mnpls.-St. Paul) 5 R01 CA 22195-08	PMA--A Cocarcinogen as a Lymphocyte Mitogen
51.	FAHL, William E. Northwestern University 5 R01 CA 25189-07	Hydrocarbon Carcinogenesis in Mouse and Human Cells
52.	FAHL, William E. Northwestern University 5 R01 CA 35514-02	Carcinogen-Transformed Human Cells--Genetic Traits
53.	FARBER, Emmanuel University of Toronto 5 R01 CA 21157-09	Pathogenesis of Liver Cancer Induced by Chemicals
54.	FARBER, John L. Hahnemann Med. Col. & Hospital of Phila. 5 R01 CA 32610-04	Hepatocarcinogenesis: A Role for Liver Necrosis

	Investigator	Project Title
55.	FAUSTO, Nelson Brown University 5 R01 CA 35249-02	RAS Activation in Liver Regeneration and Carcinogenesis
56.	FELDBERG, Ross S. Tufts University 5 R01 CA 19419-09	The Nature and Repair of a New Form of DNA Damage
57.	FINE, David New England Inst. for Life Sciences 5 R01 CA 34837-02	Analysis and Detection of Carcinogenic N-Nitrosamines
58.	FINK, Gerald R. Whitehead Inst. for Biomedical Res. 5 R01 CA 39961-02	Chemical Carcinogens and Frameshift Mutation in Yeast
59.	FISCHER, Susan M. University of Texas Sys. Can. Ctr. 1 R01 CA 34443-01A1	Role of Arachidonate Metabolites in Tumor Promotion
60.	FISHEL, Richard A. Dana Farber Cancer Institute 1 R01 CA 39092-01	Mismatch Nucleotide Repair In Escherichia Coli
61.	FISHER, Linda E. Univ. of Michigan (Ann Arbor) 5 R01 CA 33025-03	Excision Repair of Alkylated DNA
62.	FISHER, Paul B. Columbia University 5 R01 CA 35675-02	Analysis of Progression of the Transformed Phenotype
63.	FOSTER, Patricia L. Boston University 1 R01 CA 37880-01	Mechanisms of Mutagenesis by Aflatoxin and Other Agents
64.	FRAENKEL-CONRAT, Beatrice Univ. of California (Berkeley) 2 R01 CA 12316-15	Alkylation of Polynucleotides In Vitro and In Vivo
65.	FREEDMAN, Herbert A. Downstate Medical Center 5 R01 CA 29052-03	H-2 Locus and Local Tumorigenesis By Methylcholanthrene
66.	FRENKEL, Krystyna New York University 1 R01 CA 37858-01A1	Tumor Promoters Effecting Base Modification in DNA
67.	FRIEDBERG, Errol C. Stanford University 5 R01 CA 12428-15	DNA Repair and its Relationshio to Carcinogenesis
68.	GARTE, Seymour J. New York University 1 R01 CA 33874-01	Phorbol Esters a Phospholipid-CA^{++}Dependent Kinase

69.	GARTE, Seymour J. New York University 1 R01 CA 36342-01A1	Transforming Genes in Inhalation Carcinogenesis
70.	GEACINTOV, Nicholas E. New York University 5 R01 CA 20851-08	Characterization of Carcinogen-Nucleic Acid Complexes
71.	GOLDTHWAIT, David A. Case Western Reserve University 5 R01 CA 27528-05	Chemical Carcinogenesis and DNA Repair
72.	GOODMAN, Jay I. Michigan State University 5 R01 CA 30635-03	Genetic Toxicology--The Role of Non-Random Gene Damage
73.	GOULD, Michael N. Univ. of Wisconsin (Madison) 2 R01 CA 28954-04A1	Carcinogen Activation by Cultured Mammary Cells
74.	GOULD, Michael N. Univ. of Wisconsin (Madison) 2 R01 CA 30295-04	Human vs Rodent Mammary Mediated Mutagenesis Assay
75.	GREENBERGER, Joel S. Univ. of Massachusetts Med. School 7 R01 CA 39851-01	Stem Cell Age and X-Ray/Chemotherapy Leukemogenesis
76.	GRIFFITH, O. Hayes University of Oregon 2 R01 CA 11695-16	Photoelectron Microscopy of Cell Membranes
77.	GRISHAM, Joe W. Univ. of North Carolina Chapel Hill 5 R01 CA 24144-06	Toxicity in DNA Repair Deficient and Proficient Cells
78.	GRISHAM, Joe W. Univ. of North Carolina Chapel Hill 5 R01 CA 29323-05	Analysis of Tumor Progression in Liver Cells In Vitro
79.	GRISHAM, Joe W. Univ. of North Carolina Chapel Hill 5 R01 CA 32036-03	DNA Methyl Adducts: Toxicity, Mutation, & Transformation
80.	GROLLMAN, Arthur P. State Univ. of New York (Stony Brook) 2 R01 CA 17395-11	Molecular Pharmacology of Tumor and Virus Inhibitors
81.	GRUNBERGER, Dezider Columbia University (New York) 1 R01 CA 39547-01	Mechanism of Mutation Induced in Mammalian Genes
82.	GUDAS, Lorraine J. Dana Farber Cancer Institute 5 R01 CA 27953-06	Genetics/DNA Precursor Metabolism, Mutagenesis, Repair

83.	GUENGERICH, F. Peter Vanderbilt University 5 R01 CA 30907-04	Purified Human Enzymes and Carcinogen Metabolism
84.	GUPTA, Pawan K. Michigan Cancer Foundation 1 R23 CA 38844-01	Molecular Basis of Carcinogen Induced Mutagenesis
85.	GUPTA, Ramesh C. Baylor College of Medicine 2 R01 CA 30606-04	Reaction of Carcinogenic Aromatic Amines With DNA
86.	GURTOO, Hira L. Roswell Park Memorial Institute 5 R01 CA 37044-02	Genetics of PAH Metabolism: Role in Lung Cancer
87.	HAM, Richard G. University of Colorado (Boulder) 2 R01 CA 30028-04A1	Human Mammary Cell Growth and Function in Defined Media
88.	HANKINSON, Oliver Univ. of California (Los Angeles) 5 R01 CA 28868-06	Carcinogen Activation and Screening in Variant Cells
89.	HARD, Gordon C. Temple University 5 R01 CA 24216-06	Experimental Pathology of Renal Carcinogenesis
90.	HASELTINE, William A. Dana Farber Cancer Institute 5 R01 CA 26716-06	DNA Damage and Repair by Environmental Carcinogens
91.	HASELTINE, William A. Dana Farber Cancer Institute 2 R01 CA 29240-04A1	Characterization of Revertants of Xeroderma Pigmentosum
92.	HECHT, Stephen S. American Health Foundation 2 R01 CA 23901-07	Environmental Nitrosamines--Metabolism and Carcinogenesis
93.	HECHT, Stephen S. American Health Foundation 5 R01 CA 33285-03	Cocarcinogenicity of Ethanol with Nitrosamines
94.	HEIN, David W. Morehouse School of Medicine 5 R01 CA 34627-02	Pharmacogenetics of Drug and Carcinogen Metabolism
95.	HILL, Donald L. Southern Research Institute 5 R01 CA 30296-03	Carcinogen Metabolism in Sensitive and Resistant Species
96.	HITTELMAN, Walter N. University of Texas Sys. Can. Ctr. 5 R01 CA 27931-06	Molecular Basis of Chromosome Aberrations

97. HITTELMAN, Walter N. — Visualization of Chromosome Events in DNA Repair
University of Texas Sys. Can. Ctr.
1 R01 CA 39534-01

98. HIXSON, Douglas C. — Cellular Origins of Liver Cancer
University of Texas Sys. Can. Ctr.
1 R01 CA 37058-01

99. HNILICA, Lubomir S. — Experimental Hepatocarcinogenesis
Vanderbilt Univeristy
2 R01 CA 26412-06

100. HNILICA, Lubomir S. — Nuclear Toxicity of Heavy Metals
Vanderbilt University
1 R01 CA 36479-01A1

101. HOGAN, Michael E. — Mapping Carcinogen Binding Sites on Genes
Princeton University
1 R01 CA 39527-01

102. HOLMES, Eric H. — Fucolipid Markers During Chemical Carcinogenesis
Pacific Northwest Research Fdn.
5 R23 CA 35740-02

103. HOSEIN, Barbara H. — Human Epidermal Differentiation Reversibly Blocked by PMA
New York Blood Center
5 R23 CA 34621-03

104. HOWARD-FLANDERS, Paul — Enzymatic Repair of Damaged DNA
Yale University
5 R01 CA 26763-06

105. HUMAYUN, M. Zafri — Mutagenesis by Carcinogens: A Molecular Approach
Univ. of Medicine and Dentistry of NJ
5 R01 CA 27735-06

106. HUNT, John M. — Alloantigens as Probes for Hepatocarcinogenesis
Univ. of Texas Hlth. Sci. Ctr. (Houston)
5 R01 CA 37150-02

107. IP, Margot M. — Dietary Fat and Promotion of Mammary Carcinogenesis
Roswell Park Memorial Institute
5 R01 CA 33240-03

108. ISSENBERG, Phillip — Environmental Occurrence of Some Hydroxy Nitrosamines
University of Nebraska Med. Ctr.
5 R01 CA 29197-03

109. IVARIE, Robert D. — Inactivation of Gene Expression by DNA Alkylating Agents
University of Georgia
5 R01 CA 34066-02

110. JACOBSEN, Linda B. — Promotion and Progression of Liver Cells in Vitro
Purdue University West Lafayette
5 R01 CA 33441-03

111.	JACOBSON, Myron K. North Texas State University 2 R01 CA 23994-08	Alteration of NAD Metabolism by Chemical Carcinogens
112.	JENSON, David E. Temple University 1 R01 CA 38077-01	The Biochemistry of DNA Alkyl Phosphotriesters
113.	JENSEN, Ronald H. Univ. of California (Berkeley) 5 R01 CA 31549-03	Detection of Somatic Cell Mutations in Humans
114.	JIRTLE, Randy L. Duke University 5 R01 CA 25951-05	Survival and Carcinogenesis in Transplanted Hepatocytes
115.	JONES, Peter A. University of Southern California 5 R01 CA 39913-02	5 Azacytidine Induced Differentiation
116.	JUNGALWALA, Firoze B. Eunice Kennedy Shriver Center for Mental Retardation 5 R01 CA 16853-07	Biochemical Aspects of Experimental Brain Tumors
117.	KALLENBACH, Neville R. University of Pennsylvania 5 R01 CA 24101-07	Specificity in Frameshift Mutagenesis
118.	KAN, Lou-Sing Johns Hopkins University 5 R01 CA 27111-05	Model Alkylated Decanucleotide DNA Helices
119.	KAUFFMAN, Shirley L. Downstate Medical Center 5 R01 CA 17569-09	Lung Preneoplastic Hyperplasia and Chemical Carcinogens
120.	KAUFMAN, David G. Univ. of North Carolina Chapel Hill 5 R01 CA 20658-08	Chemical Carcinogenesis and Cell Proliferation
121.	KAUFMAN, David G. Univ. of North Carolina Chapel Hill 2 R01 CA 31733-04	Promotion of Chemical Carcinogenesis in Uterine Tissue
122.	KAUFMAN, David G. Univ. of North Carolina Chapel Hill 5 R01 CA 32238-03	Factors Influencing Initiation of Hepatocarcinogenesis
123.	KAUFMAN, David G. Univ. of North Carolina Chapel Hill 5 R01 CA 32239-03	Species Comparison of Uterine Carcinogenesis

124.	KENNEDY, Ann R. Harvard University 5 R01 CA 22704-07	Radiation and Chemical In Vitro Malignant Transformation
125.	KERR, Sylvia J. Univ. of Colorado Hlth. Sci. Ctr. 5 R01 CA 12742-11	Study of Methylations in Neoplasia
126.	KIMBALL, Paul C. Battelle Memorial Institute 5 R01 CA 33554-02	Chemical Cocarcinogenesis in the Rat: Gene Activation
127.	KLEIN-SZANTO, Andres J. University of Texas Sys. Can. Ctr. 5 R01 CA 34690-02	Importance of Dark Cells in Skin Carcinogenesis
128.	KLEIN-SZANTO, Andres J. University of Texas Sys. Can. Ctr. 5 R01 CA 35552-02	Carcinogenesis of Xenotrans-planted Human Epithelia
129.	KLEIN-SZANTO, Andres J. University of Texas Sys. Can. Ctr. 1 R01 CA 38863-01	Markers of Skin Tumor Progression
130.	KNOLL, Brian J. Univ. of Texas Hlth. Sci. Ctr. (Houston) 1 R01 CA 39792-01	Gene Expression in Liver Carcinogenesis
131.	KOESTNER, Adalbert Michigan State University 2 R01 CA 32594-03A1	Neurooncogenesis by Resorptive Carcinogens
132.	KOHEN, Elli University of Miami 7 R01 CA 39075-01	Intracellular Enzyme Kinetics and Carcinogenesis
133.	KOHWI-SHIGEMATSU, Terumi La Jolla Cancer Research Foundation 1 R01 CA 39681-01	Studies of Non-B DNA Structure with Chemical Carcinogens
134.	KRAUTER, Kenneth S. Yeshiva University 1 R01 CA 39553-01	Induction of Gene Expression by Chemical Carcinogens
135.	KRUGH, Thomas R. University of Rochester 5 R01 CA 35251-02	Mechanism of Action of Carcinogens
136.	KULESZ-MARTIN, Molly Roswell Park Memorial Institute 2 R01 CA 31101-04	Quantitative Carcinogenesis in Epithelial Cell Lines
137.	LALWANI, Narenda D. Northwestern University 1 R23 CA 38196-01	Receptors for Carcinogenic Peroxisome Proliferators

No.	Investigator / Institution / Grant	Title
138.	LAPEYRE, Jean-Numa University of Texas Sys. Can. Ctr. 5 R01 CA 31487-04	Regulation and Enzymology of DNA Methylase in Cancer
139.	LEGERSKI, Randy University of Texas Sys. Can. Ctr. 2 R01 CA 36486-02	Isolation and Cloning of Human DNA Repair Genes
140.	LEHRER, Robert I. Univ. of California (Los Angeles) 5 R01 CA 30526-03	Blood Cell Receptors for Tumor-Promoting Phorbol Esters
141.	LIEBERMAN, Michael W. Fox Chase Cancer Center 7 R01 CA 39391-01	Chemical Carcinogen-Induced DNA Repair in Human Cells
142.	LIEBERMAN, Michael W. Fox Chase Cancer Center 2 R01 CA 39392-02	Carcinogen Activation of Unexpressed Mammalian Genes
143.	LIEBERMAN, Michael W. Fox Chase Cancer Center 1 R01 CA 40263-01	Gene Expression in Carcinogen-Induced Liver Cancer
144.	LILLY, Frank Yeshiva University 5 P01 CA 31855-03	Mechanisms of Chemical Lymphomagenesis
145.	LINDAHL, Ronald G. Univ. of Alabama (University) 5 R01 CA 21103-06	Gene-Enzyme Relationship of Liver Aldehyde Dehydrogenase
146.	LING, Gilbert N. Pennsylvania Hospital 5 R01 CA 16301-10	Water in Cancer and in Normal Tissues
147.	LIPSKY, Michael M. Univ. of Maryland (Baltimore) 5 R01 CA 28951-03	Multi-Stage Renal Carcinogenesis in Rats
148.	LOEB, Lawrence A. University of Washington 5 R01 CA 24845-08	The Fidelity of DNA Replication
149.	LOMBARDI, Benito University of Pittsburgh 5 R01 CA 23449-08	Choline Deficiency, Oval Cells and Hepatocarcinogenesis
150.	LOMBARDI, Benito University of Pittsburgh 5 R01 CA 36174-02	Growth Modulatory and Promoters of Liver Cancer II
151.	MACLEOD, Michael C. University of Texas Sys. Can. Ctr. 5 R01 CA 35581-02	Specificity of Diol Epoxide: Chromatin Interactions

152. MACMANUS, John P.
Nat'l Research Council of Canada
5 R01 CA 31898-03

Incidence and Quantitation of a Tumor Protein

153. MAGUN, Bruce E.
University of Arizona
5 R01 CA 39360-02

Mechanisms of Tumor Promotion In Vivo and In Vitro

154. MAHER, Veronica M.
Michigan State University
5 R01 CA 21253-08

Interaction of Carcinogens with DNA--Repair of Lesions

155. MAHER, Veronica M.
Michigan State University
5 R01 CA 36520-02

Environmental Factors in Inherited Malignant Melanoma

156. MAHER, Veronica M.
Michigan State University
1 R01 CA 37838-01

In Vivo/In Vitro Human T-Cell Environmental Mutagenesis

157. MALKINSON, Alvin M.
University of Colorado (Boulder)
5 R01 CA 33497-03

Promotion of Lung Tumors by BHT and Glucocorticoids

158. MARCHOK, Ann C.
Oak Ridge National Laboratory
2 R01 CA 30529-04

Preneoplastic Markers in Specific Lesion Cell Populations

159. MARNETT, Lawrence J.
Wayne State University
2 R01 CA 22206-07

Studies on Malondialdehyde and Related Compounds

160. MAY, William S.
Johns Hopkins University
1 R23 CA 37903-01

Phorbol Ester Regulation of Transferrin Receptors

161. McCORMICK, J. Justin
Michigan State University
2 R01 CA 21289-07

In Vitro Transformation of Human Cells by Carcinogens

162. MEEHAN, Thomas D.
Univ. of California (San Francisco)
7 R01 CA 40598-01

Physical Interactions of BAP Diol Epoxides with DNA

163. MICHALOPOULOS, George K.
Duke University
2 R01 CA 30241-04

Cell Culture and Transplantation of Human Hepatocytes

164. MILLER, Elizabeth C.
Univ. of Wisconsin (Madison)
5 P01 CA 22484-08

Biochemical Studies in Chemical Carcinogenesis

165. MILO, George E.
Ohio State University
5 R01 CA 25907-05

Neoplastic Transformation of Human Epithelial Cells

166. MITRA, Sankar
Oak Ridge National Laboratory
5 R01 CA 31721-03
DNA Repair and Nitrosamine-Induced Carcinogenesis

167. MOORE, Peter D.
University of Illinois (Chicago)
5 R01 CA 37145-02
Replication of Damaged DNA in Mammalian Cell Extracts

168. MOSSMAN, Brooke T.
Univ. of Vermont and St. Agri. College
5 R01 CA 33501-03
Role of Minerals as Cofactors in Bronchogenic Carcinoma

169. NAKANISHI, Koji
Columbia University
5 R01 CA 11572-15
Structural and Bioorganic Studies of Bioactive Compounds

170. NGUYEN-HUU, Chi
Columbia University
5 R01 CA 37176-02
Oncogene Expression in Induced and Spontaneous Tumors

171. O'BRIEN, Thomas G.
Wistar Inst. of Anatomy and Biology
5 R01 CA 36353-02
Ionic Regulation and Tumor Promotion

172. OSSOWSKI, Liliana
Rockefeller University
5 R01 CA 08290-19
Chemotherapeutic Deoxynucleosides and Other Agents

173. OYASU, Ryoichi
Northwestern University
5 R01 CA 33511-03
Experimental Urinary Bladder Carcinogenesis

174. PALL, Martin L.
Washington State University
5 R01 CA 33503-03
Tandem Gene Duplication and Carcinogen Screening

175. PARSA, Ismail
Downstate Medical Center
5 R01 CA 30354-03
Interspecies Comparisons of Pancreas Carcinogenesis

176. PEGG, Anthony E.
Pennsylvania St. Univ.-Hershey Med. Ctr.
5 R01 CA 18137-10
Persistence of Alkylated DNA in Carcinogenesis

177. PELLING, Jill C.
University of Nebraska Med. Ctr.
1 R01 CA 40847-01
Two-Stage Skin Carcinogenesis and Altered Gene Expression

178. PENMAN, Sheldon
Massachusetts Inst. of Technology
1 R01 CA 37330-01A1
Cytoarchitecture in Tumor Promotion and Transformation

179. POUR, Parviz M.
University of Nebraska Med. Ctr.
5 R01 CA 34473-02
Improvement of a Prostatic Cancer Model

180.	PRAKASH, Satya University of Rochester 5 R01 CA 32514-03	Repair of DNA Damaged by Psoralen + 360 nm Irradiation
181.	RANDERATH, Kurt Baylor College of Medicine 5 R01 CA 32157-04	^{32}P-Labeling Test for Nucleic Acid Damage by Carcinogens
182.	RAO, M. Sambasiva Northwestern University 1 R01 CA 36043-01A1	Carcinogen-Induced Acinar Cell Lesions in Pancreas
183.	RAO, M. Sambasiva Northwestern University 1 R01 CA 36130-01A1	Gamma-Glutamyltranspeptidase Negative Hepatocarcinogenesis
184.	REDDY, Arram L. University of Washington 5 R01 CA 32716-02	Skin Tumorigenesis Studied with Cell Markers
185.	RICH, Alexander Massachusetts Inst. of Technology 5 R01 CA 29753-05	Chemical Carcinogenesis and DNA Structure
186.	RICHIE, Ellen R. University of Texas Sys. Can. Ctr. 1 R01 CA 37912-01	Mechanisms of MNU Induced Lymphoma
187.	ROGAN, Eleanor G. University of Nebraska Med. Ctr. 2 R01 CA 25176-04A2	Binding of Aromatic Hydrocarbons to Nucleic Acids
188.	ROMANO, Louis J. Wayne State University 5 R01 CA 35451-02	In Vitro Function of DNA Containing Carcinogen Adducts
189.	ROSENSTEIN, Barry S. Univ. of Texas Hlth. Sci. Ctr. (Dallas) 5 R23 CA 33920-03	Repair of 290-320 nm Induced Non-Dimer DNA Damage
190.	ROSNER, Marsha R. Massachusetts Inst. of Technology 5 R01 CA 35541-02	Modulation of Cellular Phosphorylation by Tumor Promoter
191.	ROSS, Ronald K. University of Southern California 5 R01 CA 33512-03	Hormones in the Etiology of Breast and Prostate Cancer
192.	ROSSMAN, Toby G. New York University 5 R01 CA 29258-05	Mutagenesis by Metals of Environmental Significance
193.	ROSSMAN, Toby G. New York University 5 R01 CA 35631-02	Carcinogen-Mediated Genetic Effects

194.	SARMA, D. S. University of Toronto 2 R01 CA 23958-07	DNA Repair/Replication in Chemical Carcinogenesis
195.	SARMA, D. S. University of Toronto 5 R01 CA 37077-02	Promotion of Liver Carcinogenesis by Orotic Acid
196.	SCARPELLI, Dante G. Northwestern University 5 R01 CA 34051-03	Metabolism of Pancreatic Carcinogens: Species Differences
197.	SCHUT, Herman A. Medical College of Ohio (Toledo) 5 R01 CA 30514-03	In Vitro Carcinogenesis Studies in Colon and Esophagus
198.	SEDWICK, W. David Duke University 5 R01 CA 31110-04	Antifolate-Induced Misincorporation of UDR in Human Cells
199.	SHARMA, Surendra University of Texas Sys. Can. Ctr. 5 R23 CA 38499-02	Expression of Bacterial Repair Genes in Human Cells
200	SHINOZUKA, Hisashi University of Pittsburgh 1 R01 CA 36175-01A1	Cyclosporin A, Possible Promoter of Lymphoma Induction
201.	SICILIANO, Michael J. University of Texas Sys. Can. Ctr. 5 R01 CA 34797-02	Chemotherapeutic Induction of Somatic Mutation
202.	SIRICA, Alphonse E. Virginia Commonwealth University 1 R01 CA 39225-01	Hepatic Oval Cells in Culture and In Vivo
203.	SIROVER, Michael A. Temple University 5 R01 CA 29414-05	Regulation of DNA Repair in Chemical Carcinogenesis
204.	SLAGA, Thomas J. University of Texas Sys. Can. Ctr. 5 R01 CA 34890-03	In Vitro Transformation of Epidermal Cells
205.	SMUCKLER, Edward A. Univ. of California (San Francisco) 5 R01 CA 21141-09	Pathology of Chemical Carcinogenesis
206.	SMULSON, Mark E. Georgetown University 5 R01 CA 25344-06	Carcinogens and Chromatin Structure and Function
207.	SOLT, Dennis B. Northwestern University 5 R01 CA 34160-03	Sequential Analysis of Oral Carcinogenesis

208.	SOROF, Sam Institute for Cancer Research 5 R01 CA 05945-20	Macromolecules in Chemical Carcinogenesis
209.	STAMBROOK, Peter J. University of Cincinnati 1 R01 CA 36897-01	Mammalian Cell Assay for Mutagenesis and Carcinogenesis
210.	STONER, Gary D. Medical College of Georgia 5 R01 CA 28950-05	Carcinogenesis Studies in Cultured Esophagus
211.	STRAUSS, Bernard S. University of Chicago 2 R01 CA 32436-04	Error Prone DNA Synthesis and Oncogene Mutagenesis
212.	STUART, Robert K. Johns Hopkins University 5 R01 CA 30491-03	Tumor Promoters and Regulation of Hematopoiesis
213.	SUDILOVSKY, Oscar Case Western Reserve University 1 R01 CA 35362-01A2	DNA Content of Dysplastic Lesions in Human and Rat Liver
214.	TEEBOR, George W. New York University 2 R01 CA 16669-10	Repair of Radiation-Induced Carcinogenic Damage to DNA
215.	TERZAGHI-HOWE, Margaret Oak Ridge National Laboratory 5 R01 CA 34695-03	Cell Interactions: Expression of Preneoplastic Markers
216.	TOPAL, Michael D. Univ. of North Carolina Chapel Hill 5 R01 CA 28632-05	Effects of Carcinogen Modification of DNA Precursors
217.	TROSKO, James E. Michigan State University 5 R01 CA 21104-08	Mutation and Derepression of Genes in Carcinogenesis
218.	TUKEY, Robert H. Univ. of California (San Diego) 5 R01 CA 37139-02	Cytochrome P-450 Genes and Chemical Carcinogenesis
219.	VARSHAVSKY, Alexander J. Massachusetts Inst. of Technology 5 R01 CA 33297-03	Gene Amplification and Tumor Promotion
220.	VERMA, Ajit K. Univ. of Wisconsin (Madison) 5 R01 CA 35368-02	Ca^{2+}-Dependent Processes Involved in Phorbol Ester Tumor Promotion
221.	VOLSKY, David J. University of Nebraska Med. Ctr. 5 R01 CA 33386-03	Epstein-Barr Virus and Tumor Promotion

222.	WALBORG, Earl F., Jr. University of Texas Sys. Can. Ctr. 5 R01 CA 27377-06	Membrane Glycoproteins During Hepatocarcinogenesis
223.	WALDSTEIN, Evelyn A. Tel Aviv University 5 R01 CA 35895-02	Regulation of Induced O^6-Methyl-Guanine Repair in Cells
224.	WALKER, Graham C. Massachusetts Inst. of Technology 5 R01 CA 21615-09	Mutagenesis and Repair of DNA
225.	WEBBER, Mukta M. Univ. of Colorado Hlth. Sci. Ctr. 5 R01 CA 33169-02	Intrinsic & Extrinsic Tumor Promoters in Prostate Cancer
226.	WEBER, Wendell W. University of Michigan (Ann Arbor) 1 R01 CA 39018-01	N-Acetylation Pharmacogenetics: Arylamines and DNA Damage
227.	WEINSTEIN, I. Bernard Columbia University 5 P01 CA 21111-09	Molecular Events in Chemical Carcinogenesis
228.	WEINSTEIN, I. Bernard Columbia University 2 R01 CA 26056-06	Cellular and Biochemical Effects of Tumor Promoters
229.	WEISBURGER, John H. American Health Foundation 5 R01 CA 30658-03	Strain Differences in Carcinogenesis
230.	WENDER, Paul A. Stanford University 5 R01 CA 31841-05	Synthetic Studies on Tumor Promoters and Inhibitors
231.	WENNER, Charles, E. Roswell Park Memorial Institute 5 R01 CA 13784-12	The Effect of Cocarcinogens on Cellular Membranes
232.	WETTERHAHN, Karen E. Dartmouth College 5 R01 CA 34869-02	Interaction of Chromate with Mitochondria
233.	WHALEN, Dale L. Univ. of Maryland (Baltimore Co. Campus) 2 R01 CA 17278-07A1	Kinetic Studies of Aryl Epoxide Reactions
234.	WHITLOCK, James P., Jr. Stanford University 5 R01 CA 32786-03	Carcinogen-Metabolizing Enzymes: Action in Variant Cells
235.	WILLIAMS, Jerry R. Johns Hopkins University 7 R01 CA 39531-01	Cellular Hypermutability in Cancer Promotion

No.	Investigator / Institution / Grant	Title
236.	WINKLE, Stephen A. Rutgers State University 5 R01 CA 34762-03	Cooperative, Selective Carcinogen, Drug Binding to DNA
237.	WITSCHI, Hanspeter R. Oak Ridge National Laboratory 5 R01 CA 33795-03	Enhancement of Lung Tumor Formation: Cell Kinetics
238.	WITZ, Gisela Rutgers Medical School 5 R01 CA 33270-02	Free Radicals in Tumor Promotion
239.	YAGER, James D., Jr. Dartmouth College 5 R01 CA 36701-02	Role of Gonadal Steroids in Hepatocarcinogenesis
240.	YAGER, James D., Jr. Dartmouth College 5 R01 CA 36713-03	DNA Sequence Changes During Hepatocarcinogenesis
241.	YANG, Nien-Chu C. University of Chicago 5 R01 CA 10220-15	Chemistry of Biologically Active Oxiranes
242.	YU, Fu-Li Rockford School of Medicine 2 R01 CA 30093-04A1	Aflatoxin B1 and Nucleolar RNA Synthesis
243.	ZURLO, Joanne Dartmouth College 5 R23 CA 36782-02	Inducible DNA Repair in Pancreatic Carcinogenesis

SUMMARY REPORT

SMOKING AND HEALTH

The Smoking and Health component within the Chemical and Physical Carcinogenesis Branch includes 12 grants with FY85 funding of $2.01 million and two contracts with FY85 funding of $0.40 million. It continues to support research directed toward understanding and mitigating the deleterious effects of smoking on health. Significant past efforts have included development of practical techniques for making and testing less hazardous cigarettes, identifying related diseases, and chemical analyses of major whole smoke components and their subsequent metabloic products. A major finding to date, is that low tar, low nicotine cigarette smoke is less harmful to experimental animals than high tar, high nicotine cigarette smoke. These findings are reflected in the current trend to low tar, low nicotine commercial cigarettes by the consumer. Current program activities are focused on the toxicological and pharmacological aspects of the problem, with emphasis on nicotine and nicotine metabolites.

Grant and Contract Activity Summary:

During the past several years, there has been an increase in the numbers of brands of low tar/low yield cigarettes on the U.S. market. As a result of many smokers switching to these newer brands and in some cases at least, changing their smoking patterns, there is an unanswered question regarding "compensation" by the smoker when such a change is made. There is a real need for scientific evidence regarding the consequences, if any, of switching to lower tar and nicotine cigarettes. Little information is available on the changes in the daily cigarette consumption, in the aggressiveness of puff profiles, in the levels of nicotine and cotinine in blood plasma, and in the carbon monoxide in the blood and expired air. These are the factors that have given rise, during the past several years, to the widespread controversy related to cigarette switching. No convincing evidence of the extent of such changes is now available from the limited studies that have taken place. Millions of smokers today are aware of the health consequences of smoking. Yet, because of their well-established smoking habit, they will not or cannot quit smoking at this time. Their position is similar to that of many alcohol and drug abusers. Without strong evidence one way or the other, specialists who help these smokers could be providing misleading or even harmful guidance. For example, should such a smoker remain on his/her current brand, or is it preferable to switch to a lower tar and nicotine brand, possibly as an intermediate sequential step towards smoking cessation? Scientific evidence on the effects of switching would provide answers for these kinds of questions. Consequently, such information would be of significant importance to all researchers in the smoking and health field, to physicians counseling patients who smoke, to public health officials, and, of course, to the general smoking population. Two studies are presently being conducted which should yield quantitative data on the question of compensation (11) (12).

One study involves seventy-two volunteers (36 men, 36 women), who currently smoke cigarette brands yielding 14-17 mg of tar and corresponding yields of nicotine. The volunteers will switch sequentially to five lower yield brands. Each alternative brand will be smoked for a two-week period. Plasma samples for cotinine and nicotine assay, expired air samples for CO determination and COHb estimation, and Respitrace parameters for puff profile analyses are to be the main data to be obtained from this study. The volunteers will provide acceptability ratings for

each alternative brand and will be contacted two months after the experiment to determine if each participant returned to his or her original brand and if not what brand was chosen for regular use.

In a second study, work was conducted to determine if the amount of CO absorbed by a smoker is influenced by the type of cigarette that is smoked (e.g., ventilated vs unventilated) and the manner in which the person smokes the cigarette. In a series of three experiments, the relationships were established between CO boost and particular topography variables: puff volume, inhalation volume (the amount of smoke drawn into the lungs), and breathhold duration (the amount of time that the inhalation volume was held in the lungs). In each of the experiments, one of the aforementioned variables was manipulated while the others were held constant. Subjects were able to puff and inhale according to the experimental protocols since auditory feedback was provided which told them when to stop puffing, when to stop inhaling, and when to start exhaling. Initial results indicate that CO boost varies as a function of puff volume, is influenced to a lesser extent by breathhold duration, and is not affected by inhalation depths ranging from 20-60% of a smoker's vital capacity. These studies are significant in that the relationship between smoking topographies and biological exposure levels can be experimentally determined. This should yield data which can be interpreted easily concerning the interrelationship between behavioral and biological measures of smoking.

Several projects are being supported which are directed toward understanding the pharmacological role of nicotine and Tobacco Specific N-Nitrosamines (TSNA) in tobacco products. Recent results from one project (3) has demonstrated that enzyme activated 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) was able to methylate guanine residues of DNA. Furthermore a correlation was established between the presence of O^6-methylguanine (O^6-MeGua) in tissues of NNK treated rats and the susceptibility of these tissues to the carcinogenic properties of NNK. Thus the presence and persistence of O^6-MeGua in the rat target tissues could play a role in the induction of tumors by NNK. Considering the relatively high level of NNK in tobacco smoke, snuff, and chewing tobacco, and the high carcinogenic potency of NNK in rodents, the results obtained suggest that the formation of O^6-MeGua and other NNK-DNA adducts might be crucial events in the initiation of tobacco related cancer. Previous studies have demonstrated some similarities between the metabolism of N'-nitrosonornicotine (NNN) and NNK and some of the DNA adducts formed by these two N-nitrosamines are likely to have identical structures. In order to study the persistence of those DNA adducts in individuals exposed actively or passively to tobacco smoke and to be able to define groups of tobacco smokers, snuff dippers, or tobacco chewers at high risks of developing cancer, it is necessary to develop an analytical technique able to measure sub-femtomole amounts of adducts in microgram samples of DNA. Such developmental studies are currently underway.

Another study (6) is involved with the endogenous formation of N-nitrosamines in tobacco users. In one experiment, 15 nonsmoking and 15 smoking men were placed on a controlled low-proline diet for 12 days. On days 1 through 3 of the study, these volunteers received the standard diet, on days 4 through 6, this diet was supplemented by a daily dose of 300 mg of proline, on days 7 through 9 the proline supplement was maintained and 1 g of ascorbic acid was also given each day. Twenty-four hour urine samples were collected on days 3, 6 and 9. The mean 24 hr. urinary excretion of N-nitrosoproline in nonsmokers was 3.55, 3.61 and 4.68 micrograms and in cigarette smokers 5.90 micrograms ($p = 0.47$), 11.7 micrograms

(p = 0.031), and 4.56 micrograms (p = 0.46). These findings demonstrated a significant potential of the inhaled smoke for endogenous N-nitrosation.

In another experiment, four nonsmokers volunteered for a research program involving passive smoke exposure and a controlled dietary regimen. The non-smokers' average urinary nitrosoproline excretion of days 1 and 2 of the study (control days) were 2.75 and 3.20 micrograms/24 hr. Passive smoke exposure on days 3 and 4 was effected by keeping the volunteers in a $16m^3$ chamber constantly polluted by the sidestream of 4 smouldering cigarettes puffed intermittently by the vacuum device of a smoking machine. This treatment did not markedly affect the N-nitrosoproline excretion in the urine which averaged 2.75 micrograms and 3.88 micrograms/24 hr. Thus, under these controlled conditions, the inhalation of environmental smoking pollutants did not increase the potential for endogenous nitrosation in the probands.

During recent years, snuff dipping has become increasingly popular, especially among younger groups in the population. On the average, a snuff dipper consumes about 10 g of fine-cut chewing tobacco daily. Commercial fine-cut tobacco contains between 1.5 and 2.5% nitrate, meaning that a regular snuff dipper has daily exposure to about 200 mg of nitrate from tobacco. The bacterial flora of the saliva is likely responsible for the conversion of a significant amount of this nitrate to nitrite. Thus, snuff dipping should increase the potential for endogenous nitrosation in man which can lead to carcinogenic N-nitrosamines. This concept has been confirmed in a study with 30 nonsmoking, snuff dipping students of a military college. Both these snuff dippers and 10 students who did not use any form of tobacco, were on identical diets and had comparable physical activities. Measurement of N-nitrosoproline excretion showed significant increases in the urine of the snuff dippers (4.6 micrograms/L) over the amounts in urine of nonusers of tobacco (0.9 micrograms/L) (p = 0.018).

Additional studies (4) have focused on the carcinogenic properties of 4-(N-methyl-N-nitrosamino)1-(3-pyridinyl)-1-butanone (NNK) and its ability to methylate DNA. In order to determine the relative potency of dimethylnitrosamine (DMN) and NNK, 2 groups of F344 male rats were injected s.c. 3 times a week for 20 weeks with a solution of either DMN or NNK (0.33 mmol/kg b.w.). The experiment was terminated after 26 months (30% survival). The incidence of tumors in 26 DMN treated rats was: nasal papilloma (1), lung adenoma or carcinoma (0), hepatomas (2). In 29 NNK treated rats, the incidence of tumors was: nasal papillomas (5), nasal squamous cell carcinoma (1), lung adenomas (10), lung adenocarcinomas (2), hepatoma (1). These results indicate that NNK is a more potent nasal (p greater than 0.07) and lung (p less than 0.01) carcinogen than DMN. Two groups of 5 rats were injected s.c. with DMN or NNK (0.41 mmol/kg) and exsanguinated 4 hrs. later. DMN was not detected in blood (less than 0.037 nmol/ml) but NNK (2.3 ± 1.0 nmol/ml) and its metabolite 4-(methylnitrosamino)-1-(3-pyridyl)-buta-1-nol (18.7 ± 10.2 nmol/ml) were detected. Less than 0.5% ofthe doses remained at the injection sites. These results indicate that DMN is metabolized more rapidly than NNK in rats. In the DNA of DMN treated rats, levels of O^6-methylguanine/-guanine (micromol/mol) were 240 (nasal mucosa), 388 (liver) and levels of 7-methylguanine/guanine were 2500 (nasal mucosa) and 5790 (liver). In NNK treated rats, levels of O^6-mehtylguanine/guanine were 50 (nasal mucosa) and 34 (liver) and levels of 7-methylguanine/guanine were 2240 (nasal mucosa) and 908 (liver). These results demonstrate that factors other than DNA methylation play a role in carcinogenesis by NNK. These could include oxobutylation of DNA or inhibition of DNA repair by NNK metabolites.

Studies are continuing on the further characterization of the metabolic fate of nicotine in rodent and human liver microsomal preparations (2). An important consideration has been the demonstration of cytochrome P-450 dependent oxidation of nicotine to the reactive iminium intermediate already characterized in rabbit and rat tissues. Definitive evidence has been obtained to show that human liver microsomal preparations are effective in the catalysis of this pathway. Additional mechanistic studies using specifically monodeuterated nicotine analogs (the cis and trans C-5 nicotine-d_1 analogs) have provided clear evidence that this 2-electron oxidative pathway is mediated by a stereoselective process involving abstraction of the trans C-5 proton (or deuteron). The absence of a significant isotope effect in this reaction suggests that the rate limiting step involved in this major biotransformation step for nicotine is not the proton abstraction reaction itself but is likely to be an initial electron abstraction leading to an iminium ion radical which itself may possess alkylating potential. Human liver preparations proved to be more selective in this reaction than rabbit liver preparations, implying a higher degree of enzyme-substrate stereochemical requirements for the human enzyme systems.

A program project (5) is continuing on experimental tobacco carcinogenesis. Until 1981, a sucker control growth agent, maleic hydrazide-diethanolamine (MH-30) was used on over 70% of all U.S. grown tobacco. Residual amounts of this formulation give rise to N-nitrosodiethanolamine (NDELA), a moderately active to strong carcinogen in hamsters and rats. In order to monitor the expected decrease of the presence of NDELA in commercial tobacco products, several brands of products are analyzed each year for this chemical. The reduction at the present time is as much as 63% from that seen in analyses prior to 1981.

It is expected that within one to two years there will be no more residue of MH-30 in the fire-cured tobacco used for fine-cut snuff since the turn-over rate is faster in the fire-cured process due to forced fermentation; therefore, there should be a drastic decrease of NDELA levels in snuff. In cigarette tobacco it may take somewhat longer to see a drastic decrease of NDELA levels since that tobacco is usually aged 3-5 years before use in manufacturing. Plans are in place to monitor the NDELA levels in U.S. tobacco products and make the data available, not only to the scientific community, but also to regulatory agencies.

Work is well underway in humans on a study of cholinergic/beta-endorphinergic reinforcement of smoking (10). Activities have been completed on the first experiment. Specifically, under conditions of minimal deprivation, nicotine was administered in high nicotine research cigarettes and intranasally in snuff. These two nicotine conditions were compared with a zero-nicotine cigarette and a sham-smoking condition. The results indicate that nicotine, regardless of its mode of administration, significantly increased pain awareness and pain endurance thresholds. Nicotine also increased significantly heartrate, peripheral vasoconstriction, and immunoreactive beta-endorphin in peripheral plasma. The clear demonstration of nicotine-produced antinociception sets the stage for research using non-nicotine dependent subjects (ex-smokers) for whom nicotine effects cannot be based on relief of nicotine withdrawal.

The program maintains resources for analytical chemical support through an Interagency Agreement with the Department of Energy at Oak Ridge National Laboratories in Oak Ridge, Tennessee (13). Activities carried out under this agreement involve (1) providing quality-assured data on the deliveries of selected chemical constituents by commercial and experimental cigarettes, (2) providing

validated methods for the quantitative determination of additional smoke constituents, and for the assessment of smoke composition, (3) providing sampling and monitoring services to define exposures accompanying tobacco smoke inhalation exposure experiments, and (4) providing methods and data to establish the relationship of exposures and smoking conditions to the resulting dose of smoke constituents experienced by the smoker.

Work conducted through another Interagency Agreement with the United States Department of Agriculture has been completed. Several million Low Yield Reference cigarettes have been fabricated, tested, and placed in cold storage for a resource reference. This activity was undertaken after the Surgeon General's Report of 1980 indicated that one of the chief research needs is the study of reduced tar and nicotine cigarettes by routine and frequent surveillance of current and new cigarettes for specific chemical constituents and biological activity. The reference cigarette is being used in 3 of the studies described in this report.

SMOKING AND HEALTH PROGRAM

GRANTS ACTIVE DURING FY85

Investigator/Institution/Grant Number	Title
1. BENOWITZ, Neal Univ. of California (San Francisco) 5 R01 CA 32389-03	Nicotine and Tar Intake During Cigarette Smoking
2. CASTAGNOLI, Neal Univ. of California (San Francisco) 5 R01 CA 35678-02	The Pharmacological Role of Nicotine
3. FOILES, Peter G. American Health Foundation 5 R01 CA 32391-03	Tobacco-Specific Nitrosamine: RIA for DNA-Adducts
4. HECHT, Stephen S. American Health Foundation 5 R01 CA 21393-09	Metabolism of the Carcinogen Nitrosonornicotine
5. HOFFMANN, Dietrich American Health Foundation 5 P01 CA 29580-04	Experimental Tobacco Carcinogenesis
6. HOFFMANN, Dietrich American Health Foundation 5 R01 CA 35607-02	Endogenous Formation of Nicotine Derived Nitrosamines
7. LYNCH, Cornelius J. The Franklin Institute 1 R01 CA 38640-01	Cigarette Switch Follow-up Study
8. MARSHALL, Milton Southwest Fdn. for Biomedical Research 5 R01 CA 33069-02	Carcinogen Metabolism in the Cigarette Smoking Baboon
9. McCOY, George D. Case Western Reserve University 5 R01 CA 32126-03	Role of Ethanol in the Etiology of Head and Neck Cancer
10. POMERLEAU, Ovide F. Univ. of Connecticut Health Center 5 R01 CA 38087-02	Cholinergic/B Endophinergic Effects of Tobacco Smoke
11. STITZER, Maxine L. Johns Hopkins Univ. School of Medicine 5 R01 CA 37736-02	Tobacco Yield Changes: Behavioral and Biological Effect

SMOKING AND HEALTH PROGRAM

CONTRACTS ACTIVE DURING FY 85

Investigator/Institute/Contract Number	Title
12. GORI, Gio B. The Franklin Research Institute NO1 CP 31047	Cigarette Smoke Yield and Smoker Compensation
13. GUERIN, Michael Department of Energy YO1 CP 30508	Collection, Separation, and Elucidation of the Components of Cigarette Smoke and Smoke Condensate

SUMMARY REPORT

CHEMICAL RESEARCH RESOURCES

The Chemical Research Resources program of the Branch endeavors to make available to the cancer research community those critical resources which are difficult or impossible for most investigators to obtain on their own, but which are necessary for the pursuit of studies on the chemical and physical aspects of carcinogenesis. Eight resource contracts totalling $1.31 million in FY 85 dollars presently comprise this program. There are no grants included. A major effort of this program has involved the synthesis and distribution of chemical carcinogens, derivatives, and metabolites for use as authentic research standards. Some of these compounds of major interest are available with ^{14}C or ^{3}H labeling. Tritium-labeled analogs of vitamin A, known as retinoids, which have shown promise in studies conducted for the Biological and Chemical Prevention program are also synthesized under contract and made available for pharmacologic and metabolic investigations. Also included under the resource category are two initiatives which support the Biological and Chemical Prevention program. These include a contract for the synthesis of kilogram quantities of retinoids for subsequent testing in chemoprevention and toxicity assays and a contract for the tracheal organ culture bioassay of new retinoids developed by the chemoprevention program. The Research Resources program also monitors an instrument loan program, involving NCI-owned thermal energy analyzers, which are placed in laboratories around the world for studies on the environmental occurrence and relevance of N-nitroso compounds such as nitrosamines and nitrosamides.

The Research Resources program currently has six contractors who are involved in the synthesis of compounds, either carcinogen standards or chemopreventive agents. These contractors develop suitable routes for the unequivocal organic synthesis of compounds designated by the NCI project officer. Methods are developed to produce adequate quantities of well-characterized compounds of high purity (generally greater than 98%). Compounds are analyzed by a meaningful combination of techniques to assess purity and confirm structure. These may include ultraviolet, fluorescence and/or infrared spectrometry, nuclear magnetic resonance, mass spectrometry, high pressure liquid chromatography, thin-layer chromatography, and elemental analysis.

At the American Health Foundation (1) the current contract objective is to synthesize one gram quantities of key metabolites of benzo(b)fluoranthene (B(b)F), benzo(j)fluoranthene (B(j)F), and benzo(k)fluoranthene (B(k)F) for distribution to the research community through the NCI Chemical Carcinogen Reference Standard Repository. The benzofluoranthenes are among the most prevalent of the carcinogenic environmental polynuclear aromatic hydrocarbons (PAH), but in contrast to other hydrocarbons such as benzo(a)pyrene, relatively little is known about the mechanism by which they cause cancer. It is hoped that, as these standards are made available, research will be stimulated in this area. During this report period, synthesis has been completed on the following compounds and they have been added to the stock in the Chemical Carcinogen Reference Standard Repository: (1) 6-hydroxybenzo(b)fluoranthene (2) 9,10-dihydroxy-11,12-epoxy-9,10,11,12-tetrahydrobenzo(b)fluoranthene (3) 2-hydroxybenzo(b)flouranthene (4) 11-hydroxybenzo(b)flouranthene (5) 3-hydroxybenzo(b)flouranthene and (6) 10-hydroxybenzo-(b)flouranthene. There are now a total of 14 benzoflouranthene metabolites in the inventory. The synthesis of three additional metabolites is in progress.

Since the last annual report there has been a change of contractors in two of the synthesis contracts. The organic chemistry division of the Midwest Research Institute was purchased by Eagle Picher Industries, Inc. and placed under a subsidiary named Chemsyn Science Laboratories, located in Lenexa, Kansas. One of the synthesis contracts is involved with the synthesis of NCI-selected, nonlabeled and labeled (^3H, ^{14}C) PAH derivatives other than the benzofluoranthenes (8). The parent compounds of interest for synthesis work include benz(a)anthracene, benzo-(a)pyrene, benzo(e)pyrene, cyclopenta(c,d)pyrene, chrysene, dibenzanthracene and 3-methylcholanthrene. Derivatives of the following types are prepared as research trends dictate: phenols; quinones; epoxides; dihydrodiols; diolepoxides; alkyl and hydroxyalkyl substituted parent hydrocarbons; nitro-PAH derivatives; PAH-DNA adducts; and sulfate, glucuronide, and glutathione conjugates. The Chemsyn Science Laboratories maintains a Radiochemical Repository for the NCI under this contract. Shipments of isotopically labeled PAH metabolites are prepared and monitored for shipment to authorized recipients as directed by the NCI project officer.

During the past 12 months 43 polynuclear aromatic hydrocarbon derivatives were synthesized, characterized, and shipped to the NCI Chemical Carcinogen Reference Standard Repository or, in the case of the isotopically labeled derivatives, placed in the Radiochemical Repository. These derivatives have included ^{14}C- and ^3H-labeled racemic anti- and syn-dihydrodiol epoxides of benzo(a)pyrene (BP); K-region phenols, dihydrodiols, and ^3H-labeled derivatives of indeno(1,2,3-c,d)-pyrene; non-K-region A-ring phenols, dihydrodiols, and the epoxide of 7,12-dimethylbenz(a)anthracene; methylpyrene derivatives; multifunctional derivatives of BP (e.g., 9-phenol-4,5-dihydrodiol); and re-synthesis of labeled and unlabeled BP phenols, dihydrodiols and epoxides.

From April, 1984 through March, 1985 a total of 131 samples of radiolabeled compounds and 6 samples of unlabeled compounds were shipped to 52 different investigators after authorization by the project officer and after receipt of documentation demonstrating that the user (or his institution) possessed a license from the Nuclear Regulatory Commission for handling the isotope and the quantity involved.

Companion contract efforts at Chemsyn Science Laboratories (5) and at SRI International (3) provide for the re-synthesis of PAH derivatives in order to maintain the inventory at the Repository. Once an unequivocal route has been developed and tested several times by the previously mentioned contractors, then Chemsyn Science Laboratories and SRI International provide the future re-syntheses in order to maintain a continuing supply. Each contractor has specific parent PAH compounds for which responsibility is assigned for the preparation of derivatives. A second objective for these contractors is the syntheses of compounds from other chemical classes that are needed in the Repository: nitrosamines, aromatic amines, additional parent polynuclear aromatic hydrocarbons, aflatoxins, steroid derivatives, and physiologically active natural products, to name a few. These two contracts were recompeted during the last fiscal year and in each case the incumbent contractor won the new award.

The efforts at the Chemsyn Science Laboratories (5) resulted in the synthesis or resynthesis, purification, and characterization of 11 compounds. Three of the compounds were radiolabeled and were placed in the Radiolabeled Repository operated by the contractor. The other eight compounds were shipped to the Chemical Carcinogen Reference Standard Repository at IITRI (2). The synthesis of

the following 5 compounds is in progress: (1) 7,12-Dimethylbenz(a)anthracene (B-6) (2) 7,8,12-Trimethylbenz(a)anthracene (B-5) (3) (±)-3,4-Cyclopenta(c,d)-pyrene-3,4-oxide (C-2) (4) 4-Hydroxyaminoquinoline-1-oxide-^{3}H and its unlabeled analog. Recently, the major work under the contract at SRI (3) has involved the development of the synthesis and subsequent production of fecapentaene-12, labeled and unlabeled, and the unlabeled fecapentaene-14. This goal has been accomplished and supplies of the compounds are currently available to the scientific community. In addition the synthesis of ethyl 4-hydroxybutylnitrosamine was completed and the material was shipped to the Chemical Carcinogen Reference Standard Repository.

All nonlabeled compounds prepared under the four contracts described previously are forwarded to the Chemical Carcinogen Reference Standard Repository operated for the NCI by IIT Research Institute (2). Other items of inventory are derived from surplus, re-analyzed chemicals that are tested by the National Toxicology Program (within the National Institute of Environmental Health Sciences) and other chemicals which are purchased commercially and re-analyzed. Most commercial purchases are made as a result of a need to obtain a given chemical for in vitro testing. The Repository participates in a program for the Office of the Director, Division of Cancer Etiology, in which selected chemicals are submitted as blind-coded samples for in vitro testing and subsequent evaluation as candidates for in vivo testing.

During the past year, 673 shipments were made to the research community at large. These shipments contained custom packaged samples usually with 5 to 100 milligrams of material. Samples were furnished with analytical documentation and safety data sheets. General information on the handling and disposal of carcinogens has been provided in response to inquiries. This contract enables the NCI to provide compounds for pertinent experiments in chemical carcinogenesis which could not be carried out otherwise. Carcinogenesis research has been greatly stimulated by the availability of authentic reference standards and/or substrates. This can be attested to by the volume of published accounts of research citing the NCI Chemical Carcinogen and Radiochemical Repositories (IITRI and Chemsyn Science Laboratories) as the source of materials.

On April 1, 1983 the Chemical Research Resources program introduced a user's fee, or payback system, for samples distributed under the program. A price structure was developed which includes cost centers for the chemical cost, the handling/-packaging cost and the shipping cost. Because of the great expense involved in developing a synthesis route for a new chemical, the NCI will still be significantly involved in the support of these contract efforts. The repository contractors will bill the requestors and deduct the net income from their operating costs. The NCI then covers the balance of each month's operating cost to the contractors. The amount billed under the payback system, between April 1, 1983 and March, 1985, by Chemsyn Science Laboratories (the contract was formally with Midwest Research Institute) was $52,195 and $121,729 by IITRI.

This program makes available a number of radiolabeled retinoids, in limited quantities, for research purposes in cancer chemoprevention. Most of these compounds will be used in support of biochemical, metabolic, and pharmacologic investigations related to the chemoprevention of cancer. These compounds are synthesized by SRI International. During the last year the contractor has successfully prepared new batches of all-trans-10,11-$^{3}H_2$-retinoic acid (total activity of 119 mCi, specific activity of 3.55 Ci/mmol) and all trans-10,11-$^{3}H_2$ retinyl acetate (total activity of 75 mCi, specific activity of 3.55 Ci/mmol).

All of these preparations were double-labeled in specific positions in the molecule and have higher specific activities than did previous preparations. Several attempts to prepare tritium-labeled beta-carotene by the dicarbonyl-coupling reaction were unsuccessful. The contractor is now planning to prepare tritium-labeled beta-carotene by Wittig condensation of tritium-labeled all-trans-retinaldehyde and unlabeled all-trans-retinyl phosphonium salt. Exploratory work on the preparation of tritium-labeled naphthoic acid (TTNN-$^3H_2$) is being continued. Two different approaches of synthesis are being tested for maximum utilization of tritium-labeling. Fifty shipments, containing a total of 83.4 mCi of radiolabeled retinoids, were made during the past year to 46 investigators in the USA and one each in Canada, France, Great Britain and Sweden. A total of $26,391 has been collected from this payback activity since its inception in April of 1983.

Retinoids found to have significant activity in short-term assays, such as the hamster tracheal organ culture system, need to be synthesized in larger quantities for animal experimentation. In particular, larger quantities of retinoid are necessary for studies of efficacy in anticarcinogenesis experiments and for toxicity determinations. As retinoid structures have become more complex so have these larger scale syntheses provided through a contract with Southern Research Institute (7). Retinoids synthesized during the past year include N-(all-trans-retinoyl)amino acids, (E)-4-(2-(5,6,7,8-tetrahydro-8,8-dimethyl-2-naphthyl)-propenyl)benzoic acid, (E)-4-(2-(5,6,7,8-tetrahydro-8,8-dimethyl-2-naphthyl)-propenyl)benzyl alcohol, 13-cis-retinol, all-trans-N-(4-hydroxyphenyl)retinamide, and 2E,4E,6E)-3-methyl-7-(5,6,7,8-tetrahydro-8,8-dimethyl-2-naphthyl)-2,4,6-octatrienoic acid.

The biological activity of retinoids has been determined in many short term types of assay. The most frequently employed has been the hamster tracheal organ culture bioassay which measures the intrinsic ability of retinoids to control epithelial cell differentiation. For this reason, it is believed to have significant predictive value for the potential use of a new retinoid for prevention of epithelial (and perhaps also mesenchymal) cancer. Through a contract with IIT Research Institute (6), dose-response curves are run on all experimental retinoids synthesized by contractors and grantees in the program as well as an internal, positive standard, all-trans-retinoic acid control. The assay is extremely sensitive, detecting activity at concentrations as low as 10^{-12} molar. Many retinoids of significant activity have been detected in this system for potential use in future investigations of anticarcinogenic activity.

RESEARCH RESOURCES

CONTRACTS ACTIVE DURING FY85

Investigator/Institution/Contract No	Title
1. HECHT, Stephen S. American Health Foundation N01-CP-15747	Synthesis of Derivatives of Polynuclear Aromatic Hydrocarbons
2. KEITH, James N. IIT Research Institute N01-CP-05612	Chemical Carcinogen Reference Standard Repository
3. REIST, Elmer J. SRI International N01-CP-41028	Synthesis of Selected Chemical Carcinogens
4. RHEE, Sung W. SRI International N01-CP-05601	Synthesis of Radiolabeled Retinoids for Metabolic and Pharmacologic Studies
5. RUEHLE, Paul H. Eagle Picher Industries, Inc. N01-CP-41001	Synthesis of Selected Chemical Carcinogen Standards
6. SCHIFF, Leonard J. IIT Research Institute N01-CP-31012	Bioassay of Retinoid Activity by Trachael Organ Culture System
7. SHEALY, Y. Fulmer Southern Research Institute N01-CP-26009	Synthesis of Kilogram Amounts of Retinoids for Chemoprevention and Toxicity Studies
8. WILEY, James C. Eagle Picher Industries, Inc. N01-CP-05613	Synthesis of Derivatives of Polynuclear Aromatic Hydrocarbons

ANNUAL REPORT OF

LOW LEVEL RADIATION EFFECTS BRANCH

NATIONAL CANCER INSTITUTE

October 1, 1984 through September 30, 1985

The Low Level Radiation Effects Branch (LLREB), established in response to Public Law 95-622, plans, directs and administers a program consisting of grants and contracts investigating the means by which exposure to ionizing and non-ionizing radiations, particularly at low doses or dose rates, leads to molecular and cellular events and processes resulting in mutagenesis, cell transformation, and carcinogenesis, and the dose-effect relationships resulting therefrom; directs and administers selected epidemiological studies investigating the effects of radiation exposure in humans; provides a broad spectrum of information, advice, and consultation to scientists and to institutional science management officials relative to the National Institutes of Health (NIH) and National Cancer Institute (NCI) funding and scientific review policies and procedures, preparation of grant applications, and choice of funding instruments; maintains contact with other Federal agencies and institutions and with the broader relevant scientific community to identify new and needed research in, and related to, the fields of radiation mechanisms and effects; provides NCI management with recommendations concerning funding needs, priorities, and strategies for the support of relevant research areas consistent with the current state of development of individual research elements and the promise of new initiatives; provides information, advice, and guidance to NCI management and staff on radiation-related issues; implements the mandates of Public Law 97-414, Section 7(a), and Public Law 98-542, Sections 7(a)(2) and 7(b); represents the Department of Health and Human Services on the Science Panel of the Committee on Interagency Radiation Research and Policy Coordination, which is located within the Office of Science and Technology Policy, Office of the President.

The extramural activities of the Branch are accomplished through contractual agreements with universities and other Federal agencies, and through traditional individual research grants, program project grants, and conference grants with universities and research organizations. At present the Branch administers over 80 research activities with an annual budget of nearly 13 million dollars. The program, although of insufficient size to justify sections, consists of two broad categories of research: mechanisms of radiation damage and repair, and radiation carcinogenesis. In addition, the NIH and the NCI have assigned to the Branch responsibility for the implementation of sections of two Public Laws addressing radiation-related issues emanating from Congressional policy concerns.

Section 7(a) of Public Law 97-414, the Orphan Drug Act, requires the Secretary to conduct scientific research and prepare analyses necessary to develop valid and credible (1) assessments of the risks of thyroid cancer that are associated with thyroid doses of Iodine-131; (2) methods to estimate the thyroid doses of Iodine-131 that are received by individuals from nuclear weapons fallout; and (3) assessments of the exposure to Iodine-131 that the American people received from the Nevada atmospheric nuclear bomb tests. An ad hoc working committee consisting of relevant expertise within and outside of the government, including foreign nationals, has been formed and is addressing these issues. The

committee is organized into 3 task groups addressing the risk of thyroid cancer per unit dose of Iodine-131 to the thyroid, the dose of Iodine-131 to the thyroid per unit of exposure to Iodine-131, and the development and verification of models to estimate the exposure of the American people to Iodine-131 resulting from radioactive fallout associated with atmospheric nuclear weapons tests at the Nevada Test Site. Two meetings of the full committee have been held during this year, plus eight meetings of the 3 task groups. In addition, the required dose reassessments are being carried out via two interagency agreements and a Cancer Expert. An interim report to the Congress is in preparation and research needs are being identified. A final report will not be available for several years.

Public Law 98-542 requires the Director of the NIH to conduct a review of the reliability and accuracy of scientific and technical devices and techniques which may be useful in determining previous radiation exposure (e.g., among military personnel who participated in atmospheric nuclear weapons tests conducted by the United States or in the American occupation of Hiroshima or Nagasaki, Japan), including the availability of such devices and techniques, the categories of exposed individuals for whom the use of such devices and techniques may be appropriate, and the reliability and accuracy of dose estimates which may be derived from such devices and techniques. A review is being conducted, via an interagency agreement, by working groups to address the several relevant devices and techniques (e.g., whole body counting, bioassay studies, chromosomal alterations). A report to the Congress will be prepared prior to the end of 1986.

The Mechanisms of Radiation Damage and Repair program includes, but is not limited to, studies on molecular and cellular changes resulting from exposure to radiation, DNA damage and repair following radiation exposure, the hypermutability, mutagenesis, and malignant transformation of cells exposed to ionizing and non-ionizing radiation, mutagenicity-carcinogenicity relationships following exposure to radiation, interspecies comparisons, and the interaction between cocarcinogens.

The Radiation Carcinogenesis program addresses the effects of exposure to radiation, including, for example, the role of oncogenes, studies of the sensitivity of the embryo or fetus to ionizing radiation, the effect of dose rate and linear energy transfer on radiation-induced effects, dose-effect relationships, interspecies comparisons, cocarcinogens, the incidence of selected diseases as they may relate to exposure from radioactive fallout, and synthesis of radiobiological data in the assessment of risk and the establishment of appropriate radiation protection practices.

The LLREB contributed to the support of two conferences held during the year addressing topics of relevance to the Branch: "Radiation Carcinogenesis and DNA Alterations," and "Cell Transformation in Radiobiology."

TABLE I
LOW LEVEL RADIATION EFFECTS BRANCH
(Extramural Activities - FY 1985 - Estimated)

	No. of Contracts/Grants	$ (Millions)
Research Contracts	9	2.95
Research Grants	75	9.79
Traditional Project Grants (70 grants; $8.53 million)		
Conference Grants (2 grants; $0.01 million)		
Program Project Grants (1 grant; $1.08 million)		
RFA (2 grants; $0.17 million)		
TOTAL	84	12.74

TABLE II
LOW LEVEL RADIATION EFFECTS BRANCH
(Contracts and Grants Active During FY 1985)

	FY 85 (Estimated)			
	CONTRACTS		GRANTS	
	No. of Contracts	$ (Millions)	No. of Grants	$ (Millions)
Radiation Mechanisms and Carcinogenesis	7	1.56	73	9.78
Office of the Chief	2	1.39	2	0.01
TOTAL	9	2.95	75	9.79

Research activities are concerned with a wide variety of radiation effects including mechanisms of damage and repair of DNA by ionizing and non-ionizing radiation, and radiation carcinogenesis. The majority of the 75 grants (53) support investigations relating to mechanisms of radiation damage and repair of cellular DNA, 37 of which investigate the effects of exposure to ionizing radiation and 16 of which study the consequences of exposure to ultraviolet radiation and visible light. Twenty grants and eight contracts fund studies in radiation carcinogenesis, and research addressing radiation risks and the compilation and assessment of information is supported by two grants and one contract.

Mechanisms of Radiation DNA Damage and Repair: The LLREB provides substantial support for basic research related to mechanisms of mutagenesis and malignant transformation by radiation. This information is essential for providing not only insight into the mechanisms of carcinogenesis, but also the rationale for the carcinogenic effects seen following exposure to radiation. Such studies contribute to the evaluation of risks to individuals and to populations.

A. Ionizing Radiation: Several important results have emerged in the area of mechanisms of X-ray mutagenesis. For example, a discovery of particular potential importance to the radiotherapist is the induction of hypermutability in Chinese hamster V79 cells with X-irradiation. Significantly, this phenomenon was discovered while the investigators were mimicking a sequence of clinical therapeutic treatments in cell culture; this resulted in a state of hypermutability induced in large numbers of cells, orders of magnitude higher than ordinary mutation frequencies, and persisted in cell progeny. The potential implications are that epigenetic events, e.g., heritable alterations in chromatin structure, may be important in mutation, in tumor promotion and/or progression, and by extension, as a possible risk consideration in radiotherapeutic regimens.

It is generally appreciated that cells damaged by radiation can repair at least some of the damage. An important concern for the LLREB is to determine to what extent, and with what degree of fidelity, human cells can repair following exposure to varying doses and types of radiation (e.g., high vs. low LET). In this regard the concept of "potentially lethal damage" has stimulated much work. Although it has for many years been known that proliferating cells and tissues exhibited a greater degree of radiosensitivity than did nonproliferating cells and tissue, the concept arose directly from the finding that cells in culture which are rapidly proliferating can not tolerate as much radiation as cells which are in the resting phase. This suggested the idea that in the resting phase there is more time for repair, while the proliferating phase cells tend to continue cycling and are unable to repair to the same extent as resting cells. The result is that survival is much greater in resting cells. However, when the cell medium is changed after X-irradiation, the survival of the proliferating cells is the same as that of the resting cells. This is of potential significance to the "potentially lethal damage" concept. Another study has shown in E. coli that repair of double strand breaks requires complete growth medium, whereas single strand repair proceeds rapidly with or without it. Both of these studies illustrate the importance of the environment in *in vitro* test systems.

The relationship of mutation to malignant transformation is an important concern and the end point, malignant conversion, should be carefully defined if the results are to be relevant. Often, other more convenient end points are substituted such as focus formation, agglutination, etc. In this regard anchorage independence has been a common criterion for transformation, and an investigator compared its frequency of appearance with the frequency of 6-thioguanine resistance

in baby hamster kidney cells (BHK cells) after X-irradiation. It was found that the two frequencies were essentially identical; when a radiation sensitive cell strain was tested the frequencies were again essentially the same. An addititonal finding, that the BHK cells are tumorigenic, alters the initial interpretation that a single mutation is sufficient for malignant transformation. This work supports the position that BHK cells are tumorigenic despite their classic "contact inhibition" at confluence.

A series of experiments using high-LET radiation with repair deficient and repair proficient cells demonstrated that cell DNA repair processes must be considered when formulating theories and models of cell radiosensitivity. It was shown that repair competence in cells is associated with a change in kinetics of survival, and the matched repair-deficient controls rule out possible objections as to differences in cell type, species, time in culture, etc. Other findings indicate that low dose rates of high-LET radiation suppress the effect of tumor promoters, thereby suggesting that low doses of this radiation is a promoter itself. This additional complication that high-LET radiation may be a tumor promoting agent warrants further investigation. These studies are of potential importance in high-LET radiation therapy, and in the prediction of the effects of low doses of high-LET radiation delivered at low dose rates.

In the past few years evidence has accumulated that DNA repair is more complex than anticipated and that the classical nucleotide excision pathway is integrated with other equally important mechanisms in order to maintain the integrity of the DNA. Investigators studying the preferential formation of DNA-protein cross-links and single strand DNA breaks in areas of the genome containing transcriptionally active genes found that after X-irradiation two non-histone proteins which are minor components of the nuclear matrix become covalently bound to the DNA. During the repair process the protein linked to DNA in regions of transcriptionally active areas is removed, following which other areas of the genome are bound. This dynamic interaction between the repair of DNA and the nuclear matrix suggests the coordinated involvement of three complex systems: the chromatin which contains the damaged DNA, the nuclear matrix which may "organize" the repair process, and the repair enzymes themselves. Although most work has focused on the repair enzymes to date, information now exists to study the higher levels of organization and the integrated response of these systems to radiation.

B. Ultraviolet Radiation: The LLREB is interested in ultraviolet radiation not only as a ubiquitous carcinogenic agent in the environment, but also in its plausible role as a cocarcinogen and the relationship of its repair pathways to the repair pathways for ionizing radiation. The transfection technique of DNA information transfer has become a powerful method of introducing specific genes into mammalian cells after appropriate manipulation by recombinant DNA methods. However, most human cells have proven refractory to integration of the DNA into a stable state of expression. It has recently been discovered that UV irradiation of chimeric plasmids such as pSV2-*gpt* results in an enhancement in the yield of *gpt*+ transformants when the plasmids are transfected into human cells by the calcium phosphate technique. Further, only "bulky" lesions, including pyrimidine dimers and psoralen adducts, cause this enhancement, which can be as much as 20-fold. In addition to the usefulness of this discovery to manipulate DNA in human cells in culture, these results further emphasize the need to better understand the relationships which exist between DNA repair, replication, integration, and expression following exposure to UV, and to recognize the interdependence of these processes and their response to other radiations.

Investigations concerning the response of cells to cellular and molecular damage by ultraviolet radiation have led to both conceptual and methodological advances in studies of DNA repair enzymes and pathways of repair. For example, the purification and characterization of 06-guanine methyltransferase led to the finding that it repairs monoadducts from the anticancer agent chloroethylnitrosourea which then suppresses DNA interstrand cross-link formation. This DNA interstrand cross-link formation is possibly the mechanism by which chloroethylnitrosourea manifests its therapeutic effect. It was found that resistance of human tumor xenografts to therapy with similar drugs such as BCNU correlates well with the presence of guanine methyltransferase activity in the tumor cells. Thus, the assay of the transferase activity may prove extremely useful for predicting the clinical response prior to therapy with this class of drugs.

Another investigation has divided the xeroderma pigmentosum complementation group A (XP-A) into at least two subgroups. The second subgroup is represented by a patient who is relatively resistant to UV injury. Unlike most XP-A patients who are virtually devoid of nucleotide excision repair capacity, this second subgroup has about 25% of normal capacity. Isolation and characterization of the defective enzyme may help to define the xeroderma pigmentosum group A deficiency, i.e., does the deficiency consist of the inability to incise the DNA strand near the damaged site, or is the repair system complete but lacking accessibility to the damaged site?

Other investigations address important questions about the relationship of the much less studied UV-B, UV-A, and blue portion of the spectrum to adduct formation, DNA strand breaks, transformation, and toxicity. Some provocative results include: (1) at longer wavelengths DNA-protein cross-links are an important lesion while pyrimidine dimers are not; (2) mutations at the HGPRT and ouabain loci have clearly distinct action spectra above 300nm, indicating either a different mechanism for mutation or that the targets are different; and (3) superoxide anion is generated from a range of biochemicals including NADH and NADPH contained in cells, and is of significance because the highly toxic and genome-damaging hydroxyl radical is readily derived from superoxide anion.

Radiation Carcinogenesis: The transformation of normal cells to malignant cells by ionizing and ultraviolet radiation and their subsequent expression as cancer is an undisputed fact. The relevance of this area to the LLREB program is to establish dose-effect relationships, including factors of dose rate and type of radiation; to determine whether there is a level of exposure to these agents which might be considered "safe"; to explore possibilities for intervening or ameliorating detrimental levels of exposure; and to expand the data base from which risk estimates are derived.

The risk of developing thyroid cancer following therapeutic radiation exposures is being studied in several thousand patients with a history of external irradiation to the head and neck for benign conditions during childhood. Several decades after exposure these patients are experiencing an increased risk for thyroid, salivary and neural tumors. Measurement of serum thyroglobulin appears to be an indicator of a developing tumor, with patients who had an increasing level of serum thyroglobulin being more likely to develop thyroid tumors.

The incidence of leukemia and thyroid disease in Utah is being assessed in relation to fallout from the Nevada Test Site between 1951 and 1962. Over 85% of more than 5,000 exposed and unexposed children identified and examined for thyroid cancer in the 1960s have been located, and clinical examinations of these persons,

which constitute a "thyroid cohort" study, have now begun. Subcontracts have been let to reconstruct milk production and distribution in southwestern Utah during that time in order to more effectively estimate thyroid exposure to Iodine-131 among these subjects.

Of importance in clarifying radiation-induced carcinogenesis effects are various biological and dosimetric factors influencing the expression of such effects (e.g., the relationship between the natural incidence of cancer and the susceptibility of the strain/species to radiation-induced cancers, the meaning and relevance of "dose" at the molecular and cellular level, the significance of dose rate). There is inadequate quantitative data from human populations on the relationship between the magnitude and/or temporal distribution of dose and the biological effectiveness of low-LET and high-LET radiations per unit of absorbed dose; these factors, therefore, are studied in other biological systems. For example, previous studies of tumorigenesis in rodents following low dose rate exposures indicated a reduced effectiveness in the range of 1/2 to 1/10 that of high dose rate exposures. A current study indicates that low dose rate exposures also are less effective than high dose rate exposures in causing excess tumor specific mortality in dogs. The study also shows, however, that at dose rates down to 5R/day the dog continues to be more sensitive than the mouse. These findings are indicative of the need for data from multiple animal species in the extrapolation of low dose rate effects to humans. Another study is investigating the possibility that leukemogenesis, studies of which heretofore have utilized radiation doses and dose rates that have caused perturbation of the hemopoietic stem cell pool, may be inducible without perturbation of the stem cell pool. From this study of leukemia induction in mice, it appears that protracted doses of 1.8R/day are leukemogenic and produce perturbations in the stem cell pool, while dose rates to 1.2R/day continue to affect the stem cell pool but have not yet resulted in an increase in the frequency of leukemia.

In recent years the research areas of cocarcinogenesis (i.e., the synergistic effect elicited by two carcinogenic agents) and tumor promotion have steadily converged. The original concept of tumor promotion required that the promoter not be a carcinogen itself, even at very high doses. Also, since promoters did not appear to involve mutagenesis or genomic damage, epigenetic effects were postulated to be their mechanism(s) of action. However, recent work implicating the metabolic generation of oxidants, including superoxide anion, hydrogen peroxide, and hydroxyl radicals, as the means by which the classic promoters such as phorbol esters exert their effect raises an important question. Does this mean that low levels of ionizing radiation in the environment are significantly more serious than controlled laboratory experiments have indicated? Investigations in the following systems suggest that this is potentially important: Formation of DMBA adducts and cell transformation in the hamster cheek pouch model are enhanced by low levels of X-irradiation, while acute doses diminish both the adducts and transformation; similar results have been observed in the mouse mammary tumor model, again with DMBA; and transformation of C3H10T-1/2 cells by X-rays is strongly potentiated by asbestos fibers.

The interactions of ultraviolet radiation and ionizing radiation (X-rays and fission neutrons) are being determined quantitatively in terms of production of skin cancer in hairless mice. Investigators have observed that the incidence of skin cancer may be increased if the animal is exposed to promoting agents (e.g., UV, PUVA, TPA) following exposure to doses of ionizing radiation which, of themselves, do not result in an increased incidence of skin cancer. It is thought

that the immune system may be a potential factor influencing the expression of skin cancer following the several exposure regimens.

Because of the greater sensitivity of a developing embryo and fetus, there is concern that radiation may have long-term detrimental effects not recognized in the early months after birth. The variation in sensitivity to ionizing radiation as a function of stage of development is under investigation. Although not yet complete, current data indicate a statistically significant increase in tumor incidence and mortality during the first four years of life in perinatally irradiated dogs, and also suggest an increased risk for neoplasia after perinatal irradiation. This is consistent with the findings from an earlier survey of malignant disease in childhood which suggested there was a relationship between prenatal exposure to diagnostic irradiation and subsequent development of leukemia and other cancers in children during the first 10 years of life.

Other investigations in this area, however, have found that there may be late effects not apparent in the first third or more of the life span. For example, mice irradiated in utero were initially indistinguishable from their controls, but, after more than a year, immunosuppressive effects which were dose related were found in the irradiated mice, and preliminary results suggest a higher incidence of hepatomas. On the other hand, irradiation of mice at 10 or 17 days gestation with gamma-rays (10 to 50 rad) decreases the response to TPA indicating a decreased number of "initiated" cells as compared to the unirradiated controls. These seemingly contradictory results emphasize the complexities and difficulties in comparing one system with another and one species with another.

Though it appears unlikely that oncogenes are involved in the induction or initiation of malignant transformation, there is evidence indicating a role for selection and/or progression of malignant cells. This possibility again raises the question of whether low levels of radiation exposure may, in fact, enhance the expression of these genes, thus increasing both cancer incidence and lethality. The evidence to date has been mixed and includes work in which oncogene expression seems to play no role in the x-ray induction of rat thyroid tumors, whereas there may well be a connection with chronic myelogenous leukemia in humans.

A novel approach to intervening in the x-ray induction of transformation is suggested by a group of investigators studying radiation induced, hormone enhanced tumors and their suppression by protease inhibitors. Present results show that when antipain or soybean inhibitor is added to the C3H10T-1/2 transformation system there is a significant reduction in transformation frequency. This is particularly important because these agents are non-toxic, naturally occurring and could plausibly be added to the diet as a natural preventive agent.

The LLREB also provides grant support to several national and international advisory bodies which analyze and disseminate information concerning, and provide guidance on matters pertaining to, occupational and public radiation protection issues: the National Council on Radiation Protection and Measurements (NCRP), the International Commission on Radiation Units and Measurements (ICRU), and the International Commission on Radiological Protection (ICRP). During the year the ICRP produced two publications, including reports on protection of patients in radiation therapy, and planning for protection of the public in the event of major radiation accidents. Three reports have come from the ICRU on radiation dosimetry. The NCRP has published three reports during 1985, including one on the induction of thyroid cancer by ionizing radiation.

LOW LEVEL RADIATION EFFECTS

GRANTS ACTIVE DURING FY85

	Investigator/Institution/Grant Number	Project Title
1.	ALDERFER, James L. New York State Department of Health 1 R01 CA 39027-01	Effects of Light on Nucleic Acids
2.	ANANTHASWAMY, Honnavara N. University of Texas System Cancer Center 1 R23 CA 40454-01	Relationship Between UV-Associated Antigens and Transformation
3.	BALCER-KUBICZEK, Elizabeth K. University of Maryland at Baltimore 5 R23 CA 32729-03	Oncogenesis from Low-Dose-Rate Irradiation
4.	BASES, Robert E. Yeshiva University 5 R01 CA 36492-02	X-Ray Damage and Repair of Primate Cell A DNA Sequences
5.	BEDFORD, Joel S. Colorado State University 5 R01 CA 18023-11	Dose and Time Factors in Cellular Radiosensitivity
6.	BERNHARD, William A. University of Rochester 2 R01 CA 32546-10	Solid State Radiation Chemistry of Nucleic Acid Bases
7.	BOX, Harold C. Roswell Park Memorial Institute 5 R01 CA 25027-19	Transfer Mechanisms in Irradiated Biological Systems
8.	BRENT, Thomas P. St. Jude Children's Research Hospital 2 R01 CA 14799-11	Enzymes and Reactions for Repair of DNA in Human Cells
9.	BURNS, Fredric J. New York University Medical Center 1 R13 CA 39751-01	Conference--Radiation Carcinogenesis and DNA Alteration
10.	CARDIFF, Robert D. University of California, Davis 5 R01 CA 36493-02	Radiation Activation of Oncogenes
11.	CLARKSON, JUDITH M. University of Texas System Cancer Center 5 R01 CA 24540-06	The Importance of DNA Damage and Repair for Cell Survival
12.	CLAYCAMP, Gregg H. University of Kansas 5 R01 CA 35380-03	Radiation Biochemistry of DNA Base Damage

13. CLIFTON, Kelly H.
University of Wisconsin Madison
2 R01 CA 13881-13

Radiation in Vitro-Mammary Neoplasia

14. COGGIN, Joseph H., Jr.
University of South Alabama
1 R01 CA 39698-01

Role of Oncofetal Antigens in Radiation Carcinogenesis

15. COOPER, Priscilla K.
University of California, Berkeley
5 R01 CA 32986-03

Inducible Responses to Carcinogenic DNA Damage

16. DEMPLE, Bruce
Harvard University
1 R01 CA 37831-01

Oxidative DNA Damage: Repair and Cellular Responses

17. ELKIND, Mortimer M.
Colorado State University
5 R01 CA 33585-02

Complementation of Cellular Radiation Repair Pathways

18. ELKIND, Mortimer M.
Colorado State University
5 R01 CA 33701-03

Cell Radiation Response at Low Dose Rates

19. ESSIGMANN, John M.
Massachusetts Inst. of Technology
5 R01 CA 33821-03

Biological Effects of Low Doses of Ionizing Radiation

20. EVANS, Helen H.
Case Western Reserve University
5 R01 CA 23427-06

Radiation Induced Mutagenesis and Carcinogenesis

21. EWING, David
Hahnemann Univ. School of Medicine
5 R01 CA 28932-03

Lethal Damage from 02 and OH in Irradiated Cells

22. GRIFFITHS, T. Daniel
Northern Illinois University
5 R01 CA 32579-04

DNA Replication after Insult with UV

23. GRIGGS, Henry G.
John Brown University
2 R01 CA 18809-09

Ultraviolet and Ionizing Radiation Damage

24. GUERNSEY, Duane L.
University of Iowa
5 R01 CA 36483-02

X-Irradiation Induced Oncogene in Mouse Embryo Cells

25. HALL, Eric J.
Columbia University
1 R13 CA 40312-01

Conference: Cell Transformation in Radiobiology

26. HALL, Eric J.
Columbia University
5 P01 CA 12536-14

Effects of Small Doses of Ionizing Radiation

	Investigator / Institution / Grant	Title
27.	HALL, Eric J. Columbia University 5 R01 CA 37967-02	Oncogenic Transformation and High LET Radiations
28.	HANAWALT, Philip C. Stanford University 5 R01 CA 35744-02	Molecular Basis of DNA Repair Deficiency in Xeroderma Pigmentosum
29.	HARRISON, George H. University of Maryland 1 R01 CA 40223-01	Ultrasound and Malignant Transformation in Vitro
30.	HENNER, William D. Dana-Farber Cancer Institute 5 R01 CA 35767-02	Ionizing Radiation Induced DNA Damage and Repair
31.	HUBBELL, Howard R. Hahnemann University 1 R01 CA 37020-01	Oncogenes in Chronic Myelogenous Leukemia
32.	HUBERMAN, E. University of Chicago 5 R01 CA 33974-03	Mutation-Transformation: Neutron Damage and Repair
33.	HUMPHREY, Ronald M. Univ. of Texas System Cancer Center 2 R01 CA 04484-27	DNA Repair and Recovery in the Mammalian Cell Cycle
34.	ILIAKIS, George Cleveland Clinic Foundation 1 R01 CA 39938-01	Has Cellular Repair (PLD,SLD) Common Molecular Base?
35.	KANTOR, George J. Wright State University 5 R01 CA 16477-10	Effects of Radiation on Human Cells Cultured in Vitro
36.	KENNEDY, Ann R. Harvard University 5 R01 CA 34680-03	Hormones, Radiation, and Malignant Transformation
37.	KOPELOVICH, Levy Friends Medical Science Research Center 5 R01 CA 39081-02	Effects of Gamma Rays and Neutrons on Human Mutant Cells
38.	LANGE, Christopher S. Downstate Medical Center 1 R01 CA 39045-01	Radiosensitivity Prognosis Based on DNA Repair Assay
39.	LAWRENCE, David A. Albany Medical College 5 R01 CA 35889-03	Radiation Immunobiology of Carcinogenesis
40.	LETT, John T. Colorado State University 5 R01 CA 10714-16	Repair of Radiation Damage to Cellular DNA

	Investigator / Institution / Grant	Title
41.	LITTLE, John B. Harvard University 5 R01 CA 11751-16	Effects of Radiation on Stationary Cells
42.	LITTLE, John B. Harvard University 5 R01 CA 34037-03	Radiation Mutagenesis in Human Cells
43.	LURIE, Alan G. University of Connecticut Health Center 5 R01 CA 32991-03	Low-Dose X-Radiation Effects on DMBA-DNA Interactions
44.	MAHLUM, D. Dennis Pacific Northwest Laboratories 5 R01 CA 35912-03	Prenatal Irradiation and Postnatal Tumor Response
45.	MAYS, Charles W. University of Utah 5 R01 CA 28314-06	Reducing Cancer Risk by Radionuclide Chelation
46.	MCCORMICK, J. Justin Michigan State University 5 R01 CA 32924-03	Transformation of Human Cells by Ionizing Radiation
47.	MERUELO, Daniel New York University 5 R01 CA 35482-02	Cloning & Study of a Major Gene Involved in Oncogenesis
48.	NAIRN, Rodney S. University of Texas System Cancer Center 5 R01 CA 36361-02	Repair and Recombination in Radiation Sensitive Cells
49.	OLEINICK, Nancy L. Case Western Reserve University 2 R01 CA 15378-12	Radiation Induced Modifications in Protein Synthesis
50.	PEAK, Meyrick J. University of Chicago 5 R01 CA 34492-02	Solar UV Damage in Human Cells
51.	PEAK, Meyrick J. University of Chicago 5 R01 CA 37848-02	Biological Effects of Solar-UV-Generated Oxygen Species
52.	PETROVICH, Zbigniew University of Southern California 7 R01 CA 38370-01	UV-X-Ray Interaction: Mutation and Transformation
53.	REDPATH, John L. University of California, Irvine 5 R01 CA 27561-05	Photoreactivation of X-Ray Induced Damage in E. Coli
54.	ROSSI, Harald H. Columbia University 2 R01 CA 15307-12	Cell Irradiations with Molecular Ions

55.	SCHNEIDER, Arthur B. Michael Reese Hospital & Med. Ctr. 2 R01 CA 21518-09	Radiation-Induced Thyroid Cancer
56.	SCOTT, Walter A. University of Miami 5 R01 CA 35244-03	Radiation Induced Genetic Alterations in Mammalian Cells
57.	SILVERSTONE, Allen E. Sloan-Kettering Inst. Ca. Res. 1 R01 CA 37705-01	In Vitro Culture of Radiation Induced Preleukemic Cell
58.	SINCLAIR, Warren K. National Council on Radiation Protection 5 R01 CA 18001-19	Radiation Protection and Measurements
59.	SMITH, Kendric C. Stanford University 5 R01 CA 02896-29	Repair of Radiation-Induced Lesions in DNA
60.	SMITH, Kendric C. Stanford University 5 R01 CA 06437-24	Molecular Basis of Radiation Lethality
61.	SMITH, Kendric C. Stanford University 5 R01 CA 33738-03	Ionizing Radiation Mutagenesis in E. Coli
62.	SOWBY, F. David Intl. Com. on Rad. Protection 2 R01 CA 30163-04	Recommendations on Radiation Protection
63.	STEVENS, Reggie H. University of Iowa 5 R01 CA 30967-03	Late Effects of in Utero Iodine-131 Exposure
64.	SUTHERLAND, Betsy M. Associated Univ./Brookhaven Natl. Lab. 5 R01 CA 26492-06	UV Transformation, DNA Repair in Human Cells and Skin
65.	TODD, Paul W. Pennsylvania State University 5 R01 CA 35370-03	Human Somatic Cell Mutagenesis by Gamma Rays
66.	ULLRICH, Robert L. Union Carbide Corp./Oak Ridge Natl. Lab. 5 R01 CA 27531-05	Carcinogenic Interactions of Radiation and Chemicals
67.	VERMA, Surendra P. New England Medical Center, Inc. 5 R01 CA 36195-02	Membrane Composition and Radiation Damage
68.	WALDREN, Charles A. Eleanor Roosevelt Inst. for Cancer Res. 5 R01 CA 36447-02	Cell Genetic Damage at Low Doses and Dose Rates

	Investigator/Institution/Grant Number	Title
69.	WALLACE, Susan S. New York Medical College 5 R01 CA 33657-04	Repair of DNA Damage Induced by Ionizing Radiation
70.	WALLACE, Susan S. New York Medical College 5 R01 CA 35580-02	Ionizing Radiation and Transposon Mobility
71.	WARD, John F. Univ. of California, San Diego 5 R01 CA 26279-06	Mechanisms in Shouldered Survival Curves
72.	WARD, John F. Univ. of California, San Diego 5 R01 CA 28640-05	Cellular Repair of Potentially Mutagenic Damage
73.	WEISS, Herbert Sloan-Kettering Inst. Ca. Res. 5 R01 CA 31677-04	Mechanisms of Radiation Damage in Cells
74.	WILLIAMS, Jerry R. Johns Hopkins University 2 R01 CA 39543-02	X-Ray Induction of Cellular Hypermutability
75.	ZAIN, Sayeeda B. University of Rochester 5 R01 CA 36432-02	Oncogenes, Oncogene Products in Radiation-Induced Tumors

CONTRACTS ACTIVE DURING FY 85

	Investigator/Institution/Contract Number	Title
76.	BENJAMIN, Stephen A. Food and Drug Administration Y01-CM-20115	Neoplasia in Beagles after Irradiation During Development
77.	BOND, Victor P. Department of Energy Y01-CO-00711	Low Level Radiation Effects
78.	BURR, William W. Oak Ridge Associated Universities Y01-CP-50512	Review of Previous Radiation Exposure Indicators
79.	CRONKITE, Eugene P. Department of Energy Y01-CO-00712	Radiation Leukemia - Prediction of Low Dose Effects
80.	FRITZ, Thomas E. Department of Energy Y01-CO-00320	Late Effects of Protracted Irradiation in Dogs

81.	FRY, R. J. Michael Department of Energy Y01-CM-10113	Co-Carcinogenesis: Ionizing and Ultraviolet Radiation
82.	LYON, Joseph L. University of Utah N01-CO-23917	Assessment of Leukemia and Thyroid Disease in Relation to Fallout in Utah
83.	STORER, John B. Department of Energy Y01-CM-20112	Extrapolation of Radiation Risk
84.	ULLRICH, Robert L. Department of Energy Y01-CM-20111	Radiation-Induced Myelogenous Leukemia

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